PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
   Chada et al.

Group Art Unit: 1632

Serial No.: 10/017,472

Examiner: Li, Qian Janice

Filed: December 7, 2001

Atty. Dkt. No.: INGN:097US

For: Methods of Treatment Involving Human
    MDA-7

APPEAL BRIEF

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APPEAL BRIEF

MS Appeal Briefs
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

Appellants hereby submit an original and three copies of this Appeal Brief to the Board of Patent Appeals and Interferences in response to the final Office Action dated June 15, 2004. The Notice of Appeal was filed on September 15, 2004 and received by the Patent and Trademark Office on September 21, 2004, as indicated on the stamped return postcard. Therefore, the deadline to file this Appeal Brief was November 21, 2004. A request for a two-month extension of time to respond is included herewith along with the required fee. This extension will bring the due date to January 21, 2005, which is within the six-month statutory period. Should such request or fee be deficient or absent, consider this paragraph such a request and authorization to withdraw the appropriate fee under 37 C.F.R. §§ 1.16 to 1.21 from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INGN:097US.

The fee for filing this Appeal Brief is $250, and is attached hereto. If the check is inadvertently omitted, or should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed material, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski L.L.P. Account No.: 50-1212/INGN:097US.

I.   REAL PARTY IN INTEREST

The real party in interest is the assignee, Introgen Therapeutics, Inc., Austin, Texas.

II.   RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III.   STATUS OF THE CLAIMS

Claims 1-67 were originally filed with the nonprovisional patent application. In response to a Restriction Requirement, Appellants elected Group I, which corresponded to claims 1-43. In response to a Species Election mailed June 3, 2003, claims 26-31 were cancelled, claim 6 was amended, and claims 68-74 were added. In response to the Office Action Dated September 30, 2003, claims 15-17 and 36 were amended and claims 75-77 added. Claims 1-25, 32-43, and 75-77 were pending as of the Final Office Action dated June 15, 2004 ("Office Action"). A copy of the claims as they existed as of the Final Office Action is provided as Appendix A, Claims Appendix.

Appellants filed a petition regarding the Restriction Requirement Dated February 23, 2003. Appellants are petitioning for the Examiner to recognize claims 1 and 36 as proper linking claims. A copy of that Petition is provided (Evidence Appendix, Exhibit 3).

IV. STATUS OF AMENDMENTS

Appellants submitted amendments on November 17, 2004 and November 18, 2004, which was prior to this appeal. Appellants submitted a Superseding Amendment on November 23, 2004 to supercede the previous amendments because the Examiner had indicated that the first two amendments had not been entered. A copy of the Superseding Amendment is enclosed (Evidence Appendix, Exhibit 1). That amendment was submitted to clarify certain claims, such as by addressing antecedent basis issues. In an Advisory Action dated December 20, 2004, the Examiner indicated the proposed amendments would not be entered (Evidence Appendix, Exhibit 2), even though these issues address 35 U.S.C. § 112, second paragraph issues.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The present invention concerns a method of inhibiting angiogenesis in a human patient in need of such treatment comprising administering to the patient an effective amount of a human melanoma differentiation antigen-7 (MDA-7) nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis. Specification page 8, lines 12-15.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1) Claims 1-4, 7-25, 32-43, and 75-77 have been rejected as lacking enablement under 35 U.S.C. §112, first paragraph.

2) Claims 1-4, 7-25, 32-42, and 75-77 have been rejected as indefinite under 35 U.S.C. §112, second paragraph.

3) Claims 1-4, 7, 8, 10-15, 24, 25, 35, 36, 42, 4, and 77 have been rejected as anticipated under 34 U.S.C. § 102(e) as anticipated by the reference of Fisher (U.S. Patent No. 6,355,622).

4)   Claims 1-4, 7-25, 35-43, and 75-77 have been provisionally rejected under 35 U.S.C. § 102(e) as anticipated by copending Application No. 09/615,154.

5)   Claims 1-4, 7-25, 35-43, and 75-77 have been rejected under 35 U.S.C. § 102(f) because the Appellant did not invent the claimed subject matter described in Application No. 09/615,154.

6)   Claims 1, 7-9, 20-23, 36-41, and 75-76 have been rejected as obvious under 35 U.S.C. § 103(a) as being obvious over Roth *et al.* (U.S. Patent No. 6,069,134) in view of Fisher (U.S. Patent No. 6,355,622).

7)   Claims 1-4, 7-25, 35-43, and 77 have been provisionally rejected as unpatenable under the judicially created doctrine of obviousness-type double patenting over claims 91-116, 125-154, 159-174 of co-pending U.S. Application No. 09/615,154.

VII.   ARGUMENT

A.   Substantial Evidence Required to Uphold Examiner's Position

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B.   Claims 1-4, 7-25, 32-43, and 75-77 Are Enabled

The Action rejects claims 1-4, 7-25, 32-43, and 75-77 as lacking enablement for distal or systemic administration of an adenoviral vector expressing fragments of MDA-7 polypeptide for treating angiogenesis-dependent tumors, though it acknowledges that intratumoral injection of a nucleic acid expressing full-length MDA-7 polypeptide or the secreted form of MDA-7 for treating angiogenesis-depending cancers is enabled. The different grounds for the rejections, which Appellants traverse, are discussed below.

1.   MDA-7 Fragments

The Action contends that the specification and prior art of record disclose the function of only a full-length MDA-7 protein with or without a secretory signal yet the claims covers any truncated MDA-7, such as fragment 182-206 of SEQ ID NO:2, and any fragment ranging from 10 to 206 contiguous amino acids of SEQ ID NO:2. It alleges that neither the specification nor the art of record teaches the structural correlation of the MDA-7 fragments with its function for inhibiting growth of tumor cells or inhibiting angiogenesis. The Action argues that the references of Bowie *et al.* (Evidence Appendix, Exhibit 4), Skolnick *et al.* (Evidence Appendix, Exhibit 5), and Rudinger (Evidence Appendix, Exhibit 6) teach that the function of a modified protein is often unpredictable. It contends that Appellants' specification does not disclose a single fragment except for the secreted form of MDA-7 that is the functional equivalent of full-length MDA-7. Appellants respectfully traverse this rejection.

For example, the Action's citation to the Skolnick reference is taken out of context with respect to the issue at hand. The Action cites Skolnick for stating 1) "Sequence-based methods for function prediction are inadequate because of the multifunctional nature. However just knowing the structure of the protein is also insufficient for prediction of multiple functional sites" (abstract)"; and 2) "Knowing a protein's three-dimensional structure is insufficient to determine its function." The entire Skolnick paper is focused on the issue of *predicting* what a protein's function *might be* when *only* sequence information is available, such as in the context of genome sequencing-type projects, where cDNA sequences are obtained. This is reflected by the title of the reference, "From genes to protein structure and function: novel applications of computational approaches in the genomic era." The Skolnick reference might be relevant if Appellants were claiming a cDNA sequence for which no utility had been established. However, this reference is not relevant to the claimed invention because a function for MDA-7 is *already* provided and this is recited in the claims.

The Action contends that "Determination of the effects of particular modifications and fragmentations are not predictable until they are actually made and used, hence resulting in a trial and error situation." Action at page 6. However, the standard for enablement is not the need for "trial and error." The test of enablement is whether the experimentation needed to practice the invention is undue. MPEP § 2164.01 (citing *Mineral Separation v. Hyde*, 242 U.S. 261, 270 (1916)). In fact, satisfaction of the enablement requirement is not precluded by the necessity of some experimentation. *See Atlas Powder Co. v. E.I. duPont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 U.S.P.Q. 409 (Fed. Cir. 1984). Therefore, even if trial and error were required to practice the invention, the Action has still not met its burden of showing that this is tantamount to requiring "undue experimentation."

The Action relies upon the reference of Rudinger to indicate that "painstaking experimental study" is required to predict the significance of particular amino acids and sequences for different aspects of biological activity. However, this reference is irrelevant to the present invention because it was published in 1976, *almost 25* years before the current application was filed. Surely this reference does not reflect the state of the art at the time the application was filed. Particularly notable is the fact that in the last 25 years, recombinant DNA technology has made something that was extremely difficult—requiring perhaps "painstaking experimental study"—25 years ago, such as cloning a gene, a trivial pursuit, as is demonstrated by the completion of the Human Genome Project in the last two years. Moreover, mutagenesis of a protein is routine experimentation because of the advances made in recombinant DNA technology. Consequently, the Rudinger reference fails to show that undue experimentation is required to practice the claimed invention.

In fact, a skilled artisan could readily prepare fragments covered by the claims and test them for function. The specification provides the cDNA sequence for MDA-7 and teaches, for example, that fragments can be generated recombinantly. Specification at pages 35-44.

Appellants respectfully note that the PTO is required, when examining a patent application, to assume that the specification complies with §112 unless it has "acceptable evidence or reasoning" to suggest otherwise. *In re Marzocchi*, 439 F.2d 220, 223-24, 169 USPQ 367, 369-370 (CCPA. 1971). Thus, the PTO must provide reasons supported by the record as a whole what the specification is not enabling. *Application of Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219-220 (CCPA 1979). Then and only then does the burden shift to the Appellants to prove that one of ordinary skill in the art could have practiced the claimed invention without undue experimentation. *In re Strahilevitz*, 668 F.2d. 1229, 1232, 212 USPQ 561, 563-64 (CCPA

1982). In this case, the cited references regarding protein sequence and structure do not shift the burden to the Appellants.

Moreover, Appellants provide additional evidence regarding an MDA-7 fragment. The Declaration of Sunil Chada ("Declaration") (Evidence Appendix, Exhibit 7) indicates that an MDA-7 polypeptide lacking the first 48 amino acids of the full-length sequence induced cell killing in melanoma cells. Declaration at ¶ 6. Moreover, an MDA-7 polypeptide lacking the first 48 amino acids but containing a sequence targeting it to the endoplasmic reticulum suppressed the growth of prostate cancer cells (PC3 cells) and human non-small cell lung carcinoma cells (H1299 cells). Declaration at ¶ 7. Therefore, in view of the foregoing arguments, Appellants respectfully request this ground for the rejection be withdrawn.

The Action cites *Genentech Inc. v. Novo Nordisk A/S*, 42 U.S.P.Q.2d 1005 (Fed. Cir. 1997) for the proposition that "when there is no disclosure of any specific starting material . . . undue experimentation is required . . . ." Action at page 6. However, this quotation is inapplicable to the present case because the Specification provides the specific starting material because the polypeptide sequence of MDA-7 is known as well as data that the full-length and secreted form achieve a "method of inhibiting angiogenesis."

A proper *prima facie* case has not been made that the claimed invention is not enabled with respect to MDA-7 fragments. For these reasons, Appellants request this ground of rejection be withdrawn.

Claims 73 and 74 Are Separately Patentable

The Action concedes that a nucleic acid expressing full-length MDA-7 polypeptide or the secreted form of MDA-7 is enabled (Action at page 4). Thus, this aspect of the rejection does not

apply to claims 73 and 74, which recite a full-length and secreted form of MDA-7. For this reason, these claims are separately patentable from the remaining claims.

2. Routes of Administration

The Action identifies three issues regarding routes of administration that indicate undue experimentation would be required to practice the invention: a) gene therapy; b) nonviral vectors; and, c) viral vectors. Appellants respectfully traverse this ground of the rejection.

a) Gene Therapy

The Action contends that the gene therapy practitioner recognized that gene therapy for angiogenesis and cancer was neither accepted nor routine and that such a person awaited significant development and guidance for its practice. The Action relies on the references of Deonarain (Evidence Appendix, Exhibit 8), Miller (Evidence Appendix, Exhibit 9), Makrides *et al.* ("Makrides") (Evidence Appendix, Exhibit 10), and Boucher *et al.* ("Boucher") (Evidence Appendix, Exhibit 11) for its position that gene targeting to desired cells and tissue has yet to become routine in the art. Deonarain is said to illustrate this point.

Moreover, the Action contends that Miller illustrates that no single vector is considered to be universally appropriate. Miller is alleged to teach that because of the underdeveloped state of vector targeting, that gene therapy, as represented by the cystic fibrosis treatment, has relied largely on localized delivery.

Furthermore, the Makrides reference was cited to provide further evidence that there is an association between the vector system chosen and the production of a therapeutic protein, which is relevant to therapeutic efficacy. The Boucher reference is cited as indicating that another element critical to the success of gene delivery is the host resistance to foreign gene transfer. It is said to be relevant because it says that host cells have an innate ability to defend themselves against the penetration of gene therapy vectors.

Overall, the Action concludes that the claims are not enabled for their full scope to the extent they cover *in vivo* applications by any means of delivery, particularly from a distal site.

Once again, a closer examination of the cited references reveals that they do not support the Action's conclusions and also, there is evidence that indicates gene therapy can be practiced according to the specification and knowledge of the skilled artisan.

The Action cites the reference of Miller as saying, "No single delivery system is likely to be universally appropriate, for instance, the requirements of gene therapy for cystic fibrosis are greatly different from those of cancer." Action at page 8, citing page 190 of Miller. By its own admission, the Action renders the next citation to Miller and the citation to Boucher irrelevant because they both involve statements relating to the treatment of cystic fibrosis, while the present invention is related to inhibiting angiogenesis.

As for the reliance on the reference of Makrides, this reference merely states that "the choice of an expression system for production of recombinant proteins depends on many factors...." However, it is not clear how this statement indicates that undue experimentation would be required to practice the invention. Moreover, this reference says nothing about the ability to express MDA-7 or any limitations there might be with its expression.

In fact, there is evidence to support the contention that the claims are enabled. In addition to the data regarding a therapeutic effect from administration of Ad-mda7 in the specification (Examples 1, 4, 6, 9, 10 and 11), there is information relating to the administration of an MDA-7-encoding plasmid in a DOTAP:cholesterol liposome to a nude mouse. In the Declaration of Sunil Chada, Dr. Chada sets forth that nude mice with tumors exhibited reduced tumor growth and reduced levels of CD31 staining after treatment with the DOTAP:Chol-*mda-7*

complex. Declaration at ¶ 9. A reduction in levels of CD31 staining is indicative of reduced vascularization, *i.e.,* inhibition of angiogenesis.

b)   Nonviral Vectors

The Deonarain reference is said to be cited as evidence for the necessity of gene targeting, particularly with respect to systemic or distal delivery. The article is relied upon for pointing out the need for methods other than viral vectors and the need for including a targeting mechanism for non-viral vector delivery.

However, there are several reasons the Deonarain reference does not support the broad conclusion that gene therapy with nonviral-vector nucleic acids is unpredictable and inefficient.

The Action's quotation from the first line of the abstract regarding "one of the main obstacles" to fulfilling the promise of gene therapy is taken out of context, because the reference goes on to say that "Viral methods of gene delivery have been studied for a number of years and are effective vectors for gene transfer." The Deonarain reference goes on to say that alternative methods are being explored because of issues relating to mutagenesis, side effects and toxicity—not targeting and expression levels.

Also, the conclusion that the Action cites from the Deonarain reference regarding targeted gene delivery being less efficient than viral gene delivery is followed by the statement, "However, under optimal conditions, enough gene product may be produced to give a therapeutic benefit (*e.g.,* suppress a phenotype of destroy a tumour)." Therefore, the use of the specific nonviral vector nucleic acid delivery method discussed in the article is not plagued with as many problems as the Action contends.

Furthermore, this reference concerns specifically one type of nonviral vector nucleic acid delivery—"ligand-targeted receptor mediated vectors for gene delivery"—as the title indicates. There are other types of nonviral vector technology, which is not discussed by the Action at all.

Some of these are discussed in the instant application, such as lipid based non-viral formulations. Specification at pages 52-53. Therefore, even if one particular type of gene therapy is still undergoing experimentation and improvement, that does not mean that the instant claims reciting a "nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells" are not enabled, particularly where the other type of gene therapy are clearly enabled.

Moreover, the concern regarding targeting and sustained expression of a gene may be less significant for a gene such as *mda-7*. As the specification indicates, MDA-7 induces apoptosis, and it selectively induces apoptosis in cancer cells, as opposed to normal cells. Specification at page 75. That MDA-7 induces apoptosis in a cell means expression of MDA-7 does not need to be sustained because once it enters the cell and induces apoptosis, that cell is no longer around. Additionally, because MDA-7 selectively induces apoptosis in cancer cells, targeting and sustained expression of MDA-7 are not the issues that they might be for many other gene therapies. Appellants contend that the Action does not raise provide credible reasons supported by the record for its contentions that undue experimentation would be required to practice the invention because it concerns gene therapy with nonviral vector-nucleic acids.

(1)   Claims 8-11 and 75-76 are separately patentable

Claims 8-11 and 75-76 concern methods using viral vectors. To this extent, the present ground of the rejection does not pertain to them. Consequently, these claims are separately patentable from the remaining claims.

c)   Viral Vectors

The Action contends that adenovirus tissue tropism in respiratory epithelium cells offers additional evidence that targeting of adenovirus may be required for gene therapy. It states that the reference of Miller teaches that adenoviral diseases are usually associated with respiratory epithelium. It contends that the issue of tissue tropism has been raised because if viral vectors are

administered systemically from a site distal from a tumor, it may require a targeting mechanism so that a sufficient amount of the vector can be localized to the site of the targeted tumor.

Appellants note that this is arguably relevant to only one particular embodiment of the claimed invention: systemic delivery of an adenovirus vector to non-lung tissue. The specification indicates that different routes of administration are contemplated depending on the location and nature of the lesion. Specification at page 54, lines 25-30. The specification indicates that for treatment of a tumor, administration intratumorally is specifically contemplated but that continuation administration may be applied when appropriate, for example, to a tumor bed. Specification at pages 55-56. This shows two things. First, the specification teaches a situation in which systemic administration can be accomplished without tissue tropism being an issue. Second, it shows that the skilled artisan was aware that different situations call for different considerations in terms of mode of administration and construct used.

Moreover, Appellants address the issue of whether adenovirus can infect other tissues. The specification of the instant application shows that adenovirus infected breast cancer cells (Example 4), in addition to lung cancer cells (Example 10).

Appellants submit that this single embodiment does not render all of the rejected claims as nonenabled. Moreover, with respect to this single embodiment, there is general evidence showing this would work.

C.   Claims 1-4, 7-25, 32-42, and 75-77 Are Definite

The Action rejects claims 1-4, 7-25, 32-42, and 75-77 under 35 U.S.C. § 112, second paragraph, as indefinite for failing to particularly point out and distinctly claim the subject matter that Appellants regard as the invention. There are different grounds for the rejections, which are

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discussed individually below. Each of these grounds means that the different rejected claims are separately patentable from one another.

1. Claim 9 Is Not Indefinite

The Action rejects claim 9 as indefinite for referring to "pfu," particularly the sentence "wherein the viral vector is administered at between 10^3 and 10^{13} pfu. Appellants contend that the skilled artisan would know this refers to an amount of virus, as opposed to a concentration because it is not set forth in those terms. Appellants believe the Examiner's problem is simply that the term "administered at" is used. In the interests of furthering prosecution, Appellants attempted to amend claim 9 in which the sentence was changed to "wherein between 10^3 and 10^{13} pfu is administered." Because the amendment was not entered, Appellants contend that the claim is clear on its face and that the skilled artisan understands the meaning and scope of the claim.

2. Claim 1 Is Not Indefinite

The Action rejects claim 1 and incomplete. It contends that it is not clear whether the goal of the method stated in the preamble has been resolved. Appellants respectfully traverse this rejection.

Claim 1 states:

> A method of inhibiting angiogenesis in a human patient in need of such treatment comprising administering to the patient an effective amount of a human melanoma differentiation antigen-7 (MDA-7) polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis.

The steps for achieving the claimed invention are clearly and fully set forth in the claim. The method requires "administering to the patient an effective amount of a human melanoma differentiation antigen-7 (MDA-7) polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis." There is no need to state whether the

goal of the method has been resolved. Appellants are unaware of any rule or caselaw indicating this and respectfully request the Examiner to provide support for this contention. Consequently, Appellants respectfully request this rejection be withdrawn because the claims comply with 35 U.S.C. § 112, second paragraph.

3. Claims 15 and 17 Can Be Amended

Claims 15 and 17 were rejected as lacking antecedent basis. The claims were amended to fix a clerical error in which they were amended to depend from a claim that did not contain a limitation to an "injection." However, the Superseding Amendment filed on November 23, 2004 (Evidence Appendix, Exhibit 1) was not entered.

Appellants contend that the skilled artisan would understand the error in claim dependency and appropriate correction will be made if prosecution is re-opened.

4. Claims 75 and 76 Have Sufficient Antecedent Basis

The Action rejects claims 75 and 76 because they recite "viral particles." It contends there is insufficient antecedent basis for this term. Appellants respectfully traverse this rejection.

The rejected claims read:

75. The method of claim 8, wherein 10^{10} to 10^{13} viral particles are administered.
76. The method of claim 75, wherein 10^{11} to 10^{12} viral particles are administered.

Claim 8, from which these claim ultimately depends says, "wherein the expression vector is a viral vector." The skilled artisan would understand that the number of viral particles recited in claims 75 and 76 refers to the amount of viral vector that is administered to a patient. The specification indicates that doses to be administered can be "described in terms of plaque forming units (PFU) or viral particles for a viral construct" and a variety of such doses are provided on the order of 10^3 to 10^{13} viral particles (including 10^{10}, 10^{11}, and 10^{12} viral particles).

Specification at page 56, lines 26-28. Thus, the term "viral vector" provides sufficient basis for the term "viral particles." Appellants respectfully request this rejection be traversed.

D.   Claims Are Novel

1.   Claims 1-4, 7, 8, 10-15, 24, 25, 35, 36, 42, and 43 Are Not Anticipated by Fisher

The Action rejects claims 1-4, 7, 8, 10-15, 24, 25, 35, 36, 42, and 43 under 35 U.S.C. §102(e) as being anticipated by Fisher (U. S. Patent No. 6, 355,622) (Evidence Appendix, Exhibit 12). Fisher is alleged to teach a method of inhibiting an angiogenesis-dependent cancer in a subject suffering from cancer comprising intratumoral administration of a replication-deficient adenoviral vector encoding the MDA-7 gene (amino acids 1-206 of SEQ ID NO:2) to nude mice bearing human cervical carcinoma cells. The Action contends that Fisher also teaches that the nucleic acid could be imbedded in liposomes introduced into the cell. Finally, it concludes that ectopic expression of MDA-7 inhibits the growth of tumor cells and may provide therapeutic benefit for the treatment of human cancer, and as such, anticipates the instant claims. Appellants respectfully traverse this rejection.

The Federal Circuit case of *Kalman v. Kimberly-Clark Corp.*, 713 F.2d 760 (Fed. Cir. 1983) states that *identity of invention* is required for anticipation. *Each element* of the claim in issue must be found in a single prior art reference. The claims recite:

> A method of inhibiting angiogenesis in a human patient in need of such treatment comprising administering to the patient an effective amount of a human melanoma differentiation antigen-7 (MDA-7) polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis.

The Fisher patent, however, does not even mention angiogenesis or inhibition of angiogenesis. Accordingly, it does not anticipate the claimed invention.

The Action notes that the elected species for a disease is an angiogenesis-dependent cancer. It also contends, "Clearly, Fisher *et al.*, teach inhibiting angiogenesis dependent cancer (*e.g.*, column 5, lines 32-43) even though they do not choose using the term 'angiogenesis,' which is a well known condition associated with cancer. For example, the specification (page 110) cites Folkman *et al* (1990), who teaches the evidence that tumors are angiogenesis dependent." Action at page 17.

However, the Examiner has provided no evidence that Fisher teaches inhibiting angiogenesis and that they simply chose not to use the term "angiogenesis."

The instant application provides specific data that the process of angiogenesis is specifically inhibited. On page 92 at lines 15-20 the specification indicates that tumors treated *in vivo* with Ad-mda7 had fewer blood vessels than untreated tumors. Furthermore, on page 99, data is provided that tumors treated with Ad-mda7 had significantly lower levels of CD31 expression, which indicates fewer blood vessels. Fisher does not teach inhibiting angiogenesis because this method was invented by the present inventors, not by Fisher *et al.*

There is no evidence presented that the data in Fisher includes inhibition of angiogenesis. This inhibition involves the endothelial cells near the tumor cell and is separate from transducing the tumor cells to promote apoptosis (though methods can involve achieving both). *See* Specification at page 23, lines 9-11 ("Administration of a nucleic acid encoding mda-7, vira viral, or nonviral vectors, to anti-angiogenic target cells, which can comprise endothelial cells, as well as the administration to tumor cells is contemplated."). The Action does not provide any support for the contention that Fisher teaches or suggests inhibiting angiogenesis with mda-7. Anticipation requires that each element of the invention be taught. Consequently, Fisher does not teach the claimed invention.

Moreover, that the invention concerns inhibiting angiogenesis in an angiogenesis-dependent cancer does not mean that Fisher's teaching of treating cancer covers the claimed invention. As discussed above, Fisher was unaware that angiogenesis could be inhibited by MDA-7 and there is no evidence that the experiments they employed resulted in inhibition of angiogenesis. Therefore, the Action fails to present a evidence to support its anticipation rejection. Appellants respectfully request this rejection be withdrawn.

2. Claims 1-4, 7-25, 35-43, 75 and 76 Are Not Anticipated by a Co-Pending Application

The Action provisionally rejects claims 1-4, 7-25, 35-43, 75, and 76 under 35 U.S.C. §102(e) over Application No. 09/615,154 ("the '154 application") (Evidence Appendix, Exhibit 13). It contends that claims in the '154 application are drawn to a method of treating a tumor in a patient comprising administering a viral vector expressing an mda-7 polypeptide or fragment with amino acids 182-206 of SEQ ID NO:2 combined with conventional chemotherapy, surgery, and radiation therapy. The Action further states that there is considerable overlap in the claims and that the inventions are co-extensive. Appellants respectfully traverse this rejection.

The case of *Kalman v. Kimberly-Clark Corp.*, 713 F.2d 760 (Fed. Cir. 1983), states that *identity of invention* is required for anticipation. *Each element* of the claim in issue must be found in a single prior art reference. The Action provides no evidence that the '154 application teaches a "method of inhibiting angiogenesis in a human patient in need of such treatment comprising administering to the patient an effective amount of a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis." For example, no evidence is provided that the '154 application teaches or suggests inhibiting angiogenesis. Consequently, there is no evidence that the instant claims are anticipated by the '154 application. In addition, with the exception of claims 21-23 (reciting that the nucleic acid is administered

before, after, or during chemotherapy, immunotherapy or radiotherapy), there is no evidence that limitations in the other dependent claims are taught by this reference.

> (1)　Claims 2-4, 7-20, 24-25, 35-43, 75 and 76 Are Separately Patentable

These claims are separately patentable from each other because no evidence is provided that the elements of these claims are taught by the '154 application. Therefore, even if the rejection of claims 1 and 23-25 were affirmed, the rejection of these claims cannot stand.

3.　Claims 1-4, 7-25, 35-43, 75 and 76 Are Not Anticipated under 102(f)

The Action rejects claims 1-4, 7-25, 35-43, 75 and 76 under 35 U.S.C. §102(f) over the '154 application. It contends that the Appellant did not invent the claimed subject matter because there is a different inventive entity between the present application and the '154 application, yet the '154 application anticipates the instantly claimed invention. Appellants respectfully traverse this rejection.

As discussed in the previous section, there is no evidence that each element of the claimed invention is suggested or taught by the cited application. This much is required for an anticipation rejection. Because there is no evidence that the '154 application provides methods of inhibiting angiogenesis, Appellants respectfully request this rejection be withdrawn.

> (1)　Claims 2-4, 7-20, 24-25, 35-43, 75 and 76 Are Separately Patentable

These claims are separately patentable from each other because no evidence is provided that the elements of these claims are taught by the '154 application. Therefore, even if the rejection of claims 1 and 23-25 were affirmed, the rejection of these claims cannot stand.

E.　Claims 1, 7-9, 20-23, 36-41, and 75-76 Are Not Obvious

The Action rejects claims 1, 7-9, 20-23, and 36-41 under 35 U.S.C. §103(a) as being unpatentable over Roth *et al.* (U. S. Patent No. 6,069,134) (Evidence Appendix, Exhibit 14) in

view of Fisher (U. S. Patent No. 6,355,622). It alleges that Roth teaches a method of administering a DNA damaging agent with an adenoviral vector expressing a tumor suppressor, particularly p53, for the treatment of cancer. The Action further contends that Fisher teaches using adenovirus encoding MDA-7 for the treatment of cancer and administering vectors to tumor cells, which may provide a therapeutic benefit for the treatment of human cancer in general. The Action acknowledges that Fisher does not discuss the details of such therapy. The Action also argues that claims 20-23 and 37-41 have limitations regarding the timing of the combination therapy that neither of the references discusses. It alleges that these limitations, however, fall within the bounds of optimization for a proper therapeutic regimen that a person of ordinary skill in the art would know. It concludes that thus it would have been obvious to one of ordinary skill in the art at the time of the invention to modify the methods taught by Roth by simply substituting p53 with MDA-7 as taught by Fisher. The ordinary skilled artisan is alleged to have been motivated to modify the claimed invention because the combined therapy would maximize the tumor treating effect of any individual therapy alone. Appellants respectfully traverse this rejection.

Three basic criteria must be met to establish a *prima facie* case of obviousness:

(1)   "there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings";

(2)   "there must be a reasonable expectation of success"; and

(3)   "the prior art reference (or references when combined) must teach or suggest all the claim limitations."

MPEP §2142. The present rejection does not meet at least two of these criteria because they do not teach or suggest all of the claim limitations and there was no reasonable expectation of success.

i)   Claim limitations not taught by the combination of references

As discussed above, there is no evidence that the Fisher patent does teaches or suggests inhibiting angiogenesis. A review of the Roth patent reveals that it too does not mention angiogenesis. The claims recite inhibition of angiogenesis and consequently, this combination of references does not teach each of the claim limitations.

ii)   No reasonable expectation of success

The issue is whether the combination of references provided to the skilled artisan a reasonable expectation of achieving the claimed invention, which is inhibition of angiogenesis by administering a nucleic acid expressing the human MDA-7 polypeptide. As neither reference discusses angiogenesis, there is no evidence that the skilled artisan would have any reason to believe that combining the teachings of the references would provide a way to inhibit angiogenesis in a patient. Accordingly, the skilled artisan had no reasonable expectation of success with respect to the claimed invention. For this reason as well, a proper *prima facie* case is lacking. Appellants respectfully request this rejection be withdrawn.

F.   Obviousness-Type Double Patenting Rejection Is Incorrect

The Action provisionally rejects claims 1-4, 7-25, 35-43, and new claim 77 of the application in view of copending U. S. Patent Application No. 09/615,154. The Action states that although the claims are not identical, they are not patentably distinct because the present application and the claims of the cited patent are each drawn to a method of treating a tumor in a patient comprising administering a viral vector expressing an mda-7 polypeptide combined with conventional chemotherapy, surgery, and radiation therapy. The Action further states that there is considerable overlap in the claims. Appellants respectfully traverse this rejection.

As discussed above, the Action provides no evidence that the claims in the '154 application teaches the method of the presently claimed invention. For example, no evidence is provided that the '154 application—or any other reference—teaches or suggests inhibiting angiogenesis. A *prima facie* case of obviousness requires that all of the elements of the rejected claims be taught by the cited reference(s). In this case, a proper case has not been made because this is lacking. Consequently, there is no evidence that the instant claims are obvious over the '154 application's claims. In addition, with the exception of claims 21-23 (reciting that the nucleic acid is administered before, after, or during chemotherapy, immunotherapy or radiotherapy), there is no evidence that limitations in the other dependent claims are taught by this reference.

1.   Claims 2-4, 7-20, 24-25, 35-43, and 75-77 Are Separately Patentable

These claims are separately patentable from each other because no evidence is provided that the elements of these claims are taught by the '154 application. Therefore, even if the rejection of claims 1 and 23-25 were affirmed, the rejection of these claims cannot stand.

VIII. CONCLUSION

For the foregoing reasons, Appellants believe all of the claims are in condition for allowance. The anticipation and obviousness rejections are improper and should be withdrawn. In addition, the claims are definite.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

Respectfully submitted,

Michael R. Kempelnek (51,888) for:

Michael R. Krawzsenek

Gina N. Shishima
Reg. No. 45,104
Attorney for Appellants

FULBRIGHT & JAWORSKI L.L.P.
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Date: December 29, 2004

APPENDIX A

CLAIMS

Claims Appendix

1. (Amendment filed but not entered yet) A method of inhibiting angiogenesis in a human patient in need of such treatment comprising administering to the patient an effective amount of a human melanoma differentiation antigen-7 (MDA-7) polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis.

2. (Original) The method of claim 1, wherein said patient exhibits an angiogenesis-related disease.

3. (Original) The method of claim 2, wherein the angiogenesis-related disease is further defined as angiogenesis-dependent cancer, a benign tumor, rheumatoid arthritis, psoriasis, an ocular angiogenic disease, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, a telangiectasia, hemophiliac joint, angiofibroma, wound granulation, cat scratch disease, an ulcer, an intestinal adhesion, atherosclerosis, scleroderma, or a hypertrophic scar.

4. (Original) The method of claim 3, wherein angiogenesis-dependent cancer is further defined as a solid tumor, leukemia, or a tumor metastasis.

5. (Withdrawn) The method of claim 3, wherein the benign tumor is further defined as a hemangioma, a neuroma, a neurofibroma, a trachoma, uterine fibroid, hamartoma, teratoma, or a pyogenic granuloma.

6. (Withdrawn) The method of claim 3, wherein the ocular angiogenic disease is further defined as diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, or Rubeosis.

7. (Original) The method of claim 1, wherein the nucleic acid is an expression vector.

8.   (Original) The method of claim 7, wherein the expression vector is a viral vector.

9.   (Amendment filed but not entered yet) The method of claim 8, wherein <u>between 10^3 and</u> <u>10^{13} pfu of</u> the viral vector is administered ~~at between 10^3 and 10^{13} pfu~~.

10.   (Original) The method of claim 8, wherein said viral vector is an adenoviral vector, a retroviral vector, a vaccinia viral vector, an adeno-associated viral vector, a polyoma viral vector, or a herpesviral vector.

11.   (Original) The method of claim 8, wherein said viral vector is an adenoviral vector.

12.   (Original) The method of claim 1, wherein said nucleic acid further comprises a CMV IE, dectin-1, dectin-2, human CD11c, F4/80, SM22 or MHC class II promoter.

13.   (Original) The method of claim 1, wherein the MDA-7 polypeptide or nucleic acid is administered to the patient by direct injection into an area in need of inhibition of angiogenesis.

14.   (Original) The method of claim 13, wherein the patient is administered multiple injections.

15.   (Amendment filed but not entered yet) The method of claim <u>13</u> [1], wherein the injection is performed locally to a disease site.

16.   (Amendment filed but not entered yet) The method of claim <u>13</u> [1], wherein the injection is performed regionally to a disease site.

17.   (Previously presented) The method of claim 1, wherein the injection is performed distally to a disease site.

18.   (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered to the patient by continuous infusion.

19.   (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered to the patient by intravenous injection.

20.   (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered prior to or after surgery.

21.   (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered before chemotherapy, immunotherapy, or radiotherapy.

22.   (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered during chemotherapy, immunotherapy, or radiotherapy.

23.   (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered after chemotherapy, immunotherapy, or radiotherapy.

24.   (Original) The method of claim 1, wherein the patient is a human.

25.   (Original) The method of claim 1, wherein the MDA polypeptide comprises amino acids from 1 to 206 of SEQ ID NO:2.

26.-31. (Cancelled)

32.   (Original) The method of claim 1, wherein the MDA polypeptide comprises amino acids from 182 to 206 of SEQ ID NO:2.

33.   (Original) The method of claim 1, wherein the MDA polypeptide comprises a secretory signal.

34. (Original) The method of claim 33, wherein the secretory signal is further defined as a positively charged N-terminal region in combination with a hydrophobic core.

35. (Original) The method of claim 1, wherein the patient is a cancer patient.

36. (Amendment filed but not entered yet) A method of inhibiting endothelial cell differentiation in a human patient comprising administering to the patient an effective amount of ~~a human MDA-7 polypeptide or~~ a nucleic acid molecule expressing the human MDA-7 polypeptide.

37. (Amendment filed but not entered yet) The method of claim 36, wherein a chemotherapeutic agent is administered prior to administration of ~~the MDA-7 polypeptide or~~ the nucleic acid molecule.

38. (Original) The method of claim 36 wherein a chemotherapeutic agent is administered after administration of the MDA-7 polypeptide or the nucleic acid molecule.

39. (Previously presented) The method of claim 37 or 38, wherein the chemotherapeutic agent is a DNA damaging agent.

40. (Original) The method of claim 39, wherein the DNA damaging agent is gamma-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), or hydrogen peroxide.

41. (Original) The method of claim 38, wherein the chemotherapeutic agent is a cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin, methotrexate, or analog or derivative variant thereof.

42. (Original) The method of claim 36, wherein the nucleic acid is comprised within a viral vector.

43. (Original) The method of claim 36, wherein the nucleic acid is comprised in a lipid composition.

68. (Withdrawn) The method of claim 32, wherein the MDA polypeptide comprises amino acids from 175 to 206 of SEQ ID NO:2.

69. (Withdrawn) The method of claim 68, wherein the MDA polypeptide comprises amino acids from 150 to 206 of SEQ ID NO:2.

70. (Withdrawn) The method of claim 69, wherein the MDA polypeptide comprises amino acids from 125 to 206 of SEQ ID NO:2.

71. (Withdrawn) The method of claim 70, wherein the MDA polypeptide comprises amino acids from about 100 to about 206 of SEQ ID NO:2.

72. (Withdrawn) The method of claim 71, wherein the MDA polypeptide comprises amino acids from 75 to 206 of SEQ ID NO:1.

73. (Withdrawn) The method of claim 72, wherein the MDA polypeptide comprises amino acids from 49 to 206 of SEQ ID NO:2.

74. (Withdrawn) The method of claim 73, wherein the MDA polypeptide comprises amino acids from 1 to 206 of SEQ ID NO:2.

75. (Previously presented) The method of claim 8, wherein 10^{10} to 10^{13} viral particles are administered.

76. (Previously presented) The method of claim 75, wherein 10^{11} to 10^{12} viral particles are administered.

77. (Previously presented) The method of claim 3, wherein the angiogenesis-dependent cancer is a hepatocarcinoma, retinoblastoma, astrocytoma, leukemia, neuroblastoma, mesothelioma, or non-small cell lung, small-cell lung, lung, head, neck, pancreatic, prostate, renal, bone, testicular, ovarian, cervical, gastrointestinal, lymphoma, brain, colon or bladder cancer.

APPENDIX B

EVIDENCE

Exhibit 1   Superseding Amendment filed November 23, 2004
Exhibit 2   Advisory Action mailed December 20, 2004
Exhibit 3   Petition from Requirement for Restriction filed December 21, 2004
Exhibit 4   Bowie *et al.*
Exhibit 5   Skolnick *et al.*
Exhibit 6   Rudinger
Exhibit 7   Declaration of Sunil Chada
Exhibit 8   Deonarain
Exhibit 9   Miller
Exhibit 10   Makrides
Exhibit 11   Boucher *et al.*
Exhibit 12   Fisher, U. S. Patent No. 6,355,622
Exhibit 13   Specification from Application SN 09/615,154
Exhibit 14   Roth, U. S. Patent No. 6,069,134

EXHIBIT 1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
     Chada et al.

Serial No.: 10/017,472

Filed: December 7, 2001

For: METHODS OF TREATMENT
     INVOLVING HUMAN MDA-7

Group Art Unit: 1632

Examiner: Li, Qian J.

Atty. Dkt. No.: INGN:097US

SUPERSEDING AMENDMENT

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

Applicants submit this Amendment in the above-referenced case. Consideration of this case in view of the amendments made below is requested.

It is believed that no fee is due; however, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INGN:097US.

Amendments to the claims are reflected in the listing of claims, which begins on page 2 of this paper.

Remarks begin on page 10 of this paper.

25476334.1

1

Listing of Claims

1. (Previously presented) A method of inhibiting angiogenesis in a human patient in need of such treatment comprising administering to the patient an effective amount of a human melanoma differentiation antigen-7 (MDA-7) polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis.

2. (Original) The method of claim 1, wherein said patient exhibits an angiogenesis-related disease.

3. (Original) The method of claim 2, wherein the angiogenesis-related disease is further defined as angiogenesis-dependent cancer, a benign tumor, rheumatoid arthritis, psoriasis, an ocular angiogenic disease, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, a telangiectasia, hemophiliac joint, angiofibroma, wound granulation, cat scratch disease, an ulcer, an intestinal adhesion, atherosclerosis, scleroderma, or a hypertrophic scar.

4. (Original) The method of claim 3, wherein angiogenesis-dependent cancer is further defined as a solid tumor, leukemia, or a tumor metastasis.

5. (Withdrawn) The method of claim 3, wherein the benign tumor is further defined as a hemangioma, a neuroma, a neurofibroma, a trachoma, uterine fibroid, hamartoma, teratoma, or a pyogenic granuloma.

6. (Withdrawn) The method of claim 3, wherein the ocular angiogenic disease is further defined as diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, or Rubeosis.

7. (Original) The method of claim 1, wherein the nucleic acid is an expression vector.

8. (Original) The method of claim 7, wherein the expression vector is a viral vector.

9. (Presently amended) The method of claim 8, wherein <u>between 10^3 and 10^{13} pfu of</u> the viral vector is administered ~~at between 10^3 and 10^{13} pfu~~.

10. (Original) The method of claim 8, wherein said viral vector is an adenoviral vector, a retroviral vector, a vaccinia viral vector, an adeno-associated viral vector, a polyoma viral vector, or a herpesviral vector.

11. (Original) The method of claim 8, wherein said viral vector is an adenoviral vector.

12. (Original) The method of claim 1, wherein said nucleic acid further comprises a CMV IE, dectin-1, dectin-2, human CD11c, F4/80, SM22 or MHC class II promoter.

13. (Original) The method of claim 1, wherein the MDA-7 polypeptide or nucleic acid is administered to the patient by direct injection into an area in need of inhibition of angiogenesis.

14. (Original) The method of claim 13, wherein the patient is administered multiple injections.

15. (Presently amended) The method of claim <u>13</u> [1], wherein the injection is performed locally to a disease site.

16. (Presently amended) The method of claim <u>13</u> [1], wherein the injection is performed regionally to a disease site.

17. (Presently amended) The method of claim <u>13</u> [1], wherein the injection is performed distally to a disease site.

18. (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered to the patient by continuous infusion.

19.   (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered to the patient by intravenous injection.

20.   (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered prior to or after surgery.

21.   (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered before chemotherapy, immunotherapy, or radiotherapy.

22.   (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered during chemotherapy, immunotherapy, or radiotherapy.

23.   (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered after chemotherapy, immunotherapy, or radiotherapy.

24.   (Original) The method of claim 1, wherein the patient is a human.

25.   (Original) The method of claim 1, wherein the MDA polypeptide comprises amino acids from 1 to 206 of SEQ ID NO:2.

26.-31. (Cancelled)

32.   (Original) The method of claim 1, wherein the MDA polypeptide comprises amino acids from 182 to 206 of SEQ ID NO:2.

33.   (Original) The method of claim 1, wherein the MDA polypeptide comprises a secretory signal.

34.   (Original) The method of claim 33, wherein the secretory signal is further defined as a positively charged N-terminal region in combination with a hydrophobic core.

35.   (Original) The method of claim 1, wherein the patient is a cancer patient.

36.   (Previously presented) A method of inhibiting endothelial cell differentiation in a human patient comprising administering to the patient an effective amount of a human MDA-7 polypeptide or a nucleic acid molecule expressing the human MDA-7 polypeptide.

37.   (Original) The method of claim 36, wherein a chemotherapeutic agent is administered prior to administration of the MDA-7 polypeptide or the nucleic acid molecule.

38.   (Original) The method of claim 36 wherein a chemotherapeutic agent is administered after administration of the MDA-7 polypeptide or the nucleic acid molecule.

39.   (Presently amended) The method of claim 37 or 38 ~~36~~, wherein the chemotherapeutic agent is a DNA damaging agent.

40.   (Original) The method of claim 39, wherein the DNA damaging agent is gamma-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), or hydrogen peroxide.

41.   (Original) The method of claim 38, wherein the chemotherapeutic agent is a cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin, methotrexate, or analog or derivative variant thereof.

42.   (Original) The method of claim 36, wherein the nucleic acid is comprised within a viral vector.

43.   (Original) The method of claim 36, wherein the nucleic acid is comprised in a lipid composition.

44.   (Withdrawn) A method for promoting an immune response in a patient comprising providing to the subject an amount of an MDA-7 polypeptide effective to induce an immune response in the patient.

45.   (Withdrawn) The method of claim 44, further comprising administering to the patient an antigen against which an immune response is promoted.

46.   (Withdrawn) The method of claim 45, wherein the antigen is a tumor antigen, microbial antigen, viral antigen, or fungal antigen.

47.   (Withdrawn) The method of claim 46, wherein the antigen is a tumor antigen.

48.   (Withdrawn) The method of claim 46, wherein the antigen is a microbial antigen.

49.   (Withdrawn) The method of claim 46, wherein the antigen is a viral antigen.

50.   (Withdrawn) The method of claim 46, wherein the antigen is a fungal antigen.

51.   (Withdrawn) The method of claim 47, wheren the tumor antigen is PSA, CEA, MART, MAGE1, MAGE 3, gp100, BAGE, GAGE, TRP-1, TRP-2, or PMSA.

52.   (Withdrawn) The method of claim 44, wherein the MDA-7 is provided to the patient by administering to the subject an expression construct comprising a nucleic acid sequence encoding at least 50 contiguous amino acids of SEQ ID NO:2, wherein the nucleic acid sequence is under the transcriptional control of a promoter.

53.   (Withdrawn) The method of claim 52, wherein the expression construct is a viral vector.

54. (Withdrawn) The method of claim 53, wherein the viral vector is an adenovirus vector, an adeno-associated virus vector, a herpesvirus vector, a retrovirus vector, a lentivirus vector, a vaccinia virus vector, or a polyoma vector.

55. (Withdrawn) The method of claim 44, wherein the antigen is provided to the patient by administering to the patient an expression construct comprising a nucleic acid sequence encoding the antigen, wherein the nucleic acid sequence is under the transcriptional control of a promoter.

56. (Withdrawn) The method of claim 44, wherein the MDA-7, antigen, or both are provided to the patient more than one time.

57. (Withdrawn) The method of claim 44, wherein the MDA-7, antigen, or both are provided to the patient intravenously, directly, intraperitoneally, regionally, systemically, or orally.

58. (Withdrawn) The method of claim 44, wherein the MDA-7 and antigen are provided to the subject at the same time.

59. (Withdrawn) A method of inducing expression of IL-6, IFNγ, or TNFα in a cell comprising administering to the cell an effective amount of an MDA-7 polypeptide or a nucleic acid expressing the MDA-7 polypeptide.

60. (Withdrawn) The method of claim 59, wherein expression of IL-6 is induced.

61. (Withdrawn) The method of claim 59, wherein expression of TNFα is induced.

62. (Withdrawn) The method of claim 59, wherein expression of IFNγ is induced.

63. (Withdrawn) The method of claim 59, wherein the cell is in a patient.

64.   (Withdrawn) A method of reducing cell damage from chemotherapy or radiotherapy in a cancer patient comprising administering to the patient an effective amount of a human MDA-7 polypeptide or a nucleic acid expressing the human MDA-7 polypeptide.

65.   (Withdrawn) The method of claim 64, wherein the MDA polypeptide or nucleic acid is administered to the patient when chemotherapy or radiotherapys is administered.

66.   (Withdrawn) The method of claim 64, wherein the MDA polypeptide or nucleic acid is administered to the patient after chemotherapy or radiotherapy is administered.

67.   (Withdrawn) The method of claim 54, wherein the MDA polypeptide or nucleic acid is administered to the patient more than one time.

68.   (Withdrawn) The method of claim 32, wherein the MDA polypeptide comprises amino acids from 175 to 206 of SEQ ID NO:2.

69.   (Withdrawn) The method of claim 68, wherein the MDA polypeptide comprises amino acids from 150 to 206 of SEQ ID NO:2.

70.   (Withdrawn) The method of claim 69, wherein the MDA polypeptide comprises amino acids from 125 to 206 of SEQ ID NO:2.

71.   (Withdrawn) The method of claim 70, wherein the MDA polypeptide comprises amino acids from about 100 to about 206 of SEQ ID NO:2.

72.   (Withdrawn) The method of claim 71, wherein the MDA polypeptide comprises amino acids from 75 to 206 of SEQ ID NO:1.

73.   (Withdrawn) The method of claim 72, wherein the MDA polypeptide comprises amino acids from 49 to 206 of SEQ ID NO:2.

74.   (Withdrawn) The method of claim 73, wherein the MDA polypeptide comprises amino acids from 1 to 206 of SEQ ID NO:2.

75.   (Previously presented) The method of claim 8, wherein 10^{10} to 10^{13} viral particles are administered.

76.   (Previously presented) The method of claim 75, wherein 10^{11} to 10^{12} viral particles are administered.

77.   (Previously presented) The method of claim 3, wherein the angiogenesis-dependent cancer is a hepatocarcinoma, retinoblastoma, astrocytoma, leukemia, neuroblastoma, mesothelioma, or non-small cell lung, small-cell lung, lung, head, neck, pancreatic, prostate, renal, bone, testicular, ovarian, cervical, gastrointestinal, lymphoma, brain, colon or bladder cancer.

REMARKS

In a telephone conference with Examiner Li on November 23, 2004, which Applicants' representative appreciates, the Examiner indicated that she had not yet considered the amendments filed on November 17, 2004 ("Amendment") and November 18, 2004 ("Supplemental Amendment"). Applicants respectfully request that neither the Amendment filed on November 17, 2004 nor the Supplemental Amendment filed in November 18, 2004 be considered. Instead, Applicants request that only the present amendment ("Superseding Amendment") be considered. This Superseding Amendment incorporates the previously filed Supplemental Amendment and part of the Amendment.

In this Superseding Amendment, claims 9, 15-17, and 39 are amended. Claim 39 has been amended to address an antecedent basis issue. Claim 9 has been amended to clarify the invention for previously reciting "administering at." Claims 15-17 were amended to address an antecedent basis issue. It is believed that these amendments place the case in better condition for appeal by reducing issues. These amendments do not introduce new matter as well. Applicants respectfully request these amendments be entered.

Applicants would also like to reiterate the impropriety of withdrawing claims 68-74 because these claims depend on the elected species. The Office Action mailed June 15, 2004 says that "the dependencies of claims 68-74 are improper because the sequences of claims 68-74 encompass rather then further limit the sequence recited in claim 32." Action at page 2. This is incorrect. Claim 32 and claims 68-74 are provided below: .

32.   The method of claim 1, wherein the MDA polypeptide comprises amino acids from 182 to 206 of SEQ ID NO:2.

68. (Withdrawn) The method of claim 32, wherein the MDA polypeptide comprises amino acids from 175 to 206 of SEQ ID NO:2.

69. (Withdrawn) The method of claim 68, wherein the MDA polypeptide comprises amino acids from 150 to 206 of SEQ ID NO:2.

70. (Withdrawn) The method of claim 69, wherein the MDA polypeptide comprises amino acids from 125 to 206 of SEQ ID NO:2.

71. (Withdrawn) The method of claim 70, wherein the MDA polypeptide comprises amino acids from about 100 to about 206 of SEQ ID NO:2.

72. (Withdrawn) The method of claim 71, wherein the MDA polypeptide comprises amino acids from 75 to 206 of SEQ ID NO:1.

73. (Withdrawn) The method of claim 72, wherein the MDA polypeptide comprises amino acids from 49 to 206 of SEQ ID NO:2.

74. (Withdrawn) The method of claim 73, wherein the MDA polypeptide comprises amino acids from 1 to 206 of SEQ ID NO:2.

Claims 68-74 are proper dependent claims because they further limit the sequence of the MDA-7 polypeptide. They successively require more and more amino acids from SEQ ID NO:2 and therefore are more limiting than the claims from which they depend. This also illustrates why a species election between them is inappropriate. If claim 32 is free of the prior art, claims 68-74 will also be necessarily free of the prior art and therefore, there is no issue regarding a search of these claims being an undue burden. A search of claim 32 will necessarily yield the art relevant to claims 68-74. For these reasons, Applicants respectfully request these claims no longer be considered withdrawn.
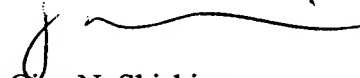
As discussed with the Examiner, Applicants do not want to amend claim 1 and claims 36-37 based on the restriction requirement, which Applicants are petitioning.

Reconsideration is respectfully requested.

CONCLUSION

Should the Examiner desire to discuss this further, she is invited to contact the undersigned attorney at 512-536-3081.

Respectfully submitted,

Gina N. Shishima
Reg. No. 45,104
Attorney for Applicants

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas   78701
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(512) 536-4598 (facsimile)

Date: November 23, 2004

EXHIBIT 2

UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/017,472	12/07/2001	Sunil Chada	INGN:097US	5209

7590   12/20/2004

Gina N. Shishima
Fulbright & Jaworski L.L.P.
Suite 2400
600 Congress Avenue
Austin, TX 78701

EXAMINER
LI, QIAN JANICE

ART UNIT	PAPER NUMBER
1632	

DATE MAILED: 12/20/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

RECEIVED
Date(s) Docketed:

DEC 2 3 2004

Client: INGN: 097US
Attorney(s): SLH, GNS
Initials:

PTO-90C (Rev. 10/03)

| Advisory Action | Application No. 10/017,472 | Applicant(s) CHADA ET AL. |
| | Examiner Q. Janice Li | Art Unit 1632 | |

-- *The MAILING DATE of this communication appears on the cover sheet with the correspondence address* --

THE REPLY FILED 23 November 2004 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE. Therefore, further action by the applicant is required to avoid abandonment of this application. A proper reply to a final rejection under 37 CFR 1.113 may <u>only</u> be either: (1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114.

PERIOD FOR REPLY [check either a) or b)]

a) ☒ The period for reply expires <u>6</u> months from the mailing date of the final rejection.

b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action, or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection. ONLY CHECK THIS BOX WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

1. ☒ A Notice of Appeal was filed on <u>21 September 2004</u>. Appellant's Brief must be filed within the period set forth in 37 CFR 1.192(a), or any extension thereof (37 CFR 1.191(d)), to avoid dismissal of the appeal.

2. ☒ The proposed amendment(s) will not be entered because:

    (a) ☒ they raise new issues that would require further consideration and/or search (see NOTE below);

    (b) ☐ they raise the issue of new matter (see Note below);

    (c) ☐ they are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or

    (d) ☐ they present additional claims without canceling a corresponding number of finally rejected claims.

    NOTE: *The amendment would raise new issues under 112, 2nd paragraph*.

3. ☐ Applicant's reply has overcome the following rejection(s): _____.

4. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).

5. ☒ The a)☐ affidavit, b)☐ exhibit, or c)☒ request for reconsideration has been considered but does NOT place the application in condition for allowance because: *See Continuation Sheet*.

6. ☐ The affidavit or exhibit will NOT be considered because it is not directed SOLELY to issues which were newly raised by the Examiner in the final rejection.

7. ☒ For purposes of Appeal, the proposed amendment(s) a)☒ will not be entered or b)☐ will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.

    The status of the claim(s) is (or will be) as follows:

    Claim(s) allowed: _____.
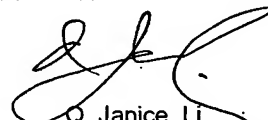
    Claim(s) objected to: _____.

    Claim(s) rejected: *1-4,7-25,32-43 and 75-77*.

    Claim(s) withdrawn from consideration: *5,6, 68-74*.

8. ☐ The drawing correction filed on _____ is a)☐ approved or b)☐ disapproved by the Examiner.

9. ☐ Note the attached Information Disclosure Statement(s)(PTO-1449) Paper No(s). _____.

10. ☐ Other: _____

Q. Janice Li
Primary Examiner
Art Unit: 1632

Continuation of 5: Applicants requested the Examiner only consider the supplemental response filed 11/23/04. In this response, Applicants reiterated the previous argument concerning the withdrawn of claims 68-74, and indicating that they are petitioning the Examiner's decision. In response, it is noted that it is applicants who elected the particular fragment of SEQ ID NO: 2 without traverse in the response filed 7/7/03. In the same response, applicants canceled claims 26-31, which are similar or the same in scope as the later submited claims 68-74. It is also noted that the claims are withdrawn only because they are drawn to non-elected species. Once the currently rejected generic claims are found allowable, a reasonable number of additional species would be considered as indicated in MPEP, "Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are writte in dependent form or otherwise include all the limitations of an allowed generic claim as provided by 37 CFR 1.141. If claims are added after the election, applicant must indicate which are readable upon the elected species. MPEP § 809.02(a)".

Further, it is noted that MPEP also teaches "Should applicant traverse on the ground that the species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidenc or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention".

Applicants did not present arguemnts to other rejections of record, thus for reasons of record, the rejections stand.

EXHIBIT 3

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Chada et al.	Group Art Unit: 1632
Serial No.: 10/017,472	Examiner: Li, Qian J.
Filed: December 7, 2001	Atty. Dkt. No.: INGN:097US
For: METHODS OF TREATMENT INVOLVING HUMAN MDA-7	

PETITION FROM REQUIREMENT FOR RESTRICTION PURSUANT TO 37 C.F.R. 1.144

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

This paper is submitted pursuant to 37 C.F.R. § 1.144 in response to the Office Communications dated February 24, 2003, and June 3, 2003.

REMARKS

Applicants are petitioning the denial that claims 1 and 36 are proper linking claims.

A. Background

In a Restriction Requirement Dated February 24, 2003, the Examiner set forth four groups for restriction. Group I stated: "Claims 1-43 are drawn to a method for inhibiting

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angiogenesis or endothelial cell differentiation comprising administering to a patient <u>a nucleic acid</u> expressing the human MDA-7 polypeptide." Exhibit A, page 2 (emphasis added). Group II stated: "Claims 1-6 and 13-41 are drawn to a method comprising administering to a patient <u>a human MDA-7 polypeptide</u>." Page 2 (emphasis added).

In the response to this Restriction filed on March 11, 2003, Applicants elected Group I and stated that "claims 1 and 36 were proper linking claims covering both Groups I and II." Exhibit B, page 2. Applicants also said, "If claim 1 or claim 36 is found to be allowable, the Group II claims must be rejoined and considered for allowance." *Id.*

In the Office Action Dated September 30, 2003, the Examiner stated:

> . . . Applicants are reminded that the restriction is not issued as linking claim type, because every invention recited in claims 1 and 36 are embraced by groups II and I. Each of the inventions requires a separate search status and consideration. The inventions are mutually exclusive and independent methods for *in vivo* gene and protein therapies. Therefore, it is <u>maintained</u> that these inventions are distinct due to their divergent subject matter.

Exhibit C, page 2. Moreover, in that Office Action, the Examiner indicated that claims 1, 13, 18-23, and 36-38 were objected to because they encompassed more than one invention as defined in the Restriction Requirement. The Action stated that claims should be amended to read upon only the elected invention. Exhibit C, page 3.

In the Response to the Office Action Dated September 30, 2003, Applicants urged reconsideration of the issue regarding claims 1 and 36 by traversing the objection to claims 1, 13, 18-23, and 36-38. Exhibit D, page 9. Applicants reiterated that claims 1 and 36 were proper linking claims. *Id.*

Now, Applicants are petitioning the restriction to the extent there has been a denial that claims 1 and 36 are proper linking claims and request the rejoinder of Group II if claim 1 or

claim 36 is found allowable. They further petition the objection to claims 1, 13, 18-23, and 36-38

as requiring amendment to reflect the election of Group I.

B.   Claims 1 and 36 Are Proper Linking Claims

Claim 1 reads:

A method of inhibiting angiogenesis in a human patient in need of such treatment comprising administering to the patient an effective amount of a human melanoma differentiation antigen-7 (MDA-7) polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis.

Claim 36 reads:

A method of inhibiting endothelial cell differentiation in a human patient comprising administering to the patient an effective amount of a human MDA-7 polypeptide or a nucleic acid molecule expressing the human MDA-7 polypeptide.

First, Applicants note that administering a polypeptide and administering a nucleic acid

expressing that polypeptide are not necessarily mutually exclusive. In fact, their relationship is

similar to that of many species, meaning that one can be used instead of the other but they are not

necessarily mutually exclusive.

Second, Applicants note that one of the most common linking claims is a "genus claim

linking species claims." MPEP § 809.03. Claims 1 and 36 are genus claims linking the different

species of administering an MDA-7 polypeptide and administering a nucleic acid molecule

expressing the human MDA-7 polypeptide. Moreover, each of these species is clearly a way of

generally providing MDA-7 polypeptide to the patient.

The linked claims "must be examined with any one of the linked inventions that may be

elected." M.P.E.P. § 814. The MPEP specifically indicates:

Where the requirement for restriction in an application is predicated upon the nonallowability of generic or other type of linking claims, applicant is entitled to

retain in the case claims to the nonelected invention or inventions. MPEP §809.04.

Therefore, it is inappropriate for the Examiner to request that any portion of the claim be amended merely based on the election of invention, as was set forth in the Response to Office Action filed on January 30, 2004. Exhibit D, page 9. In that Response, Applicants specifically indicated that withdrawal of claims was inappropriate and that they traversed the objection to claims 1, 13, 18-23, and 36-38.

Therefore, Applicants petition that claims 1 and 36 be recognized as linking claims and that Group II be re-joined if claim 1 or claim 36 is found allowable. Moreover, Applicants request clarification that Applicants need not amend the claims at this time to reflect the initial election of Group I.

It is believed that no fee is due; however, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INGN:097.

Respectfully submitted,

Gina N. Shishima
Reg. No. 45,104
Attorney for Applicants

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
(512) 474-5201
(512) 536-4598 (facsimile)

Date: December 21, 2004

<u>EXHIBIT A</u>

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/017,472	12/07/2001	Sunil Chada	INGN:097US	5209

7590   02/24/2003

Gina N. Shishima
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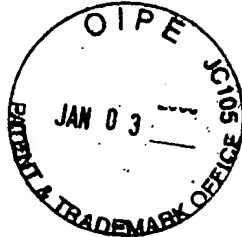
EXAMINER
LI, QIAN J

ART UNIT	PAPER NUMBER
1632	

DATE MAILED: 02/24/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

RECEIVED
Date(s) Docketed: 3/24/03 Resp.
to Rstn. Rep. Due
8/24/03 final deadline

FEB 2 7 2003

Client: INGN: 097US
Attorney(s): SLH/GNS
Initials:

1011283

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE _1_ MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.
- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
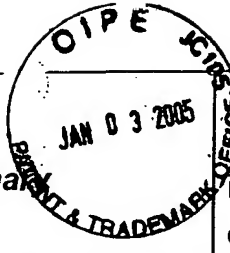- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on _12/7/01_ .

2a)☐ This action is **FINAL**.   2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) _1-67_ is/are pending in the application.

    4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) _1-67_ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

    Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

    If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

    a)☐ All b)☐ Some * c)☐ None of:

      1.☐ Certified copies of the priority documents have been received.

      2.☐ Certified copies of the priority documents have been received in Application No. _____ .

      3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

    * See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

    a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1)☐ Notice of References Cited (PTO-892)

2)☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3)☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .

4)☐ Interview Summary (PTO-413) Paper No(s). _____ .

5)☐ Notice of Informal Patent Application (PTO-152)

6)☐ Other: .

DETAILED ACTION

Election/Restrictions

1. Restriction to one of the following inventions is required under 35 U.S. C. 121:

I.   Claims 1-43 are drawn to a method for inhibiting angiogenesis or endothelial cell

        differentiation comprising administering to a patient a nucleic acid expressing the

        human MDA-7 polypeptide. Classified in Class 514, subclass 44.

II.   Claims 1-6, and 13-41 are drawn to a method comprising administering to a patient a

        human MDA-7 polypeptide. Classified in Class 514, subclass 2.

III.   Claims 44-67 are drawn to a method for promoting an immune response comprising

        administering to a patient a nucleic acid expressing the human MDA-7 polypeptide and

        an antigen. Classified in Class 514, subclass 44, and class 424, subclass 184.1.

IV.   Claims 44-51, and 56-67 are drawn to a method for promoting an immune response

        comprising administering to a patient a human MDA-7 polypeptide and an antigen.

        Classified in Class 514, subclass 2, and class 424, subclass 184.1.

2.   The inventions are distinct, each from the other because of the following reasons.

        Inventions II-IV and I are independent and distinct inventions. Inventions are distinct if

it can be shown that they are not disclosed as capable of use together and they have different

modes of operation, different functions, or different effects (MPEP § 806.04, MPEP § 808.01).

In the instant case, each of the groups I-IV are drawn to a different method of inhibiting

angiogenesis, endothelial cell differentiation, and promoting immune response. Each of the

groups differs in the starting material used in the process and mode of operation as well as

biological effects. For example, a nucleic acid has distinct biodistribution and pharmacokinetics

compared to a polypeptide, the antigens used in groups III and IV are not used in groups I and II.

Therefore, the different groups of invention require distinct technical considerations and search

criteria.

The differences of the Inventions I-IV are further underscored by their divergent

classification and independent search criteria.

Because these inventions are distinct for the reasons given above and have acquired a

separate status in the art because of their recognized divergent subject matter and different search

criteria, it would impose an undue burden to the Office if all the groups are examined together,

thus, restriction for examination purposes as indicated is proper.

3.   This application contains claims directed to the following patentably distinct species of

the claimed invention: i.e. different types of diseases that need to be treated, different viral

vectors used in the methods, different type of antigens for administration, and different

fragments of MDA-7 polypeptides. Upon election of an invention for examination in this

application, further election of a species is necessary, i.e. select a particular disease, a particular

MDA-7 fragment, and if applicable, select a particular type of antigen and a particular type of

vector for examination

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for

prosecution on the merits to which the claims shall be restricted if no generic claim is finally

held to be allowable. Currently, claims 1-67 are generic.

Applicant is advised that a reply to this requirement must include an identification of the

species that is elected consonant with this requirement, and a listing of all claims readable

thereon, including any claims subsequently added. An argument that a claim is allowable or that

all claims are generic is considered nonresponsive unless accompanied by an election.

4.   Applicant is advised that the reply to this requirement to be complete must include an

election of the invention to be examined even though the requirement be traversed (37 CFR

1.143).

Applicant is advised that where a single claim encompasses more than one invention as

defined above, upon election of an invention for examination, said claim will only be examined

to the extent that it reads upon the elected invention.

5.   Applicant is reminded that upon the cancellation of claims to a non-elected invention, the

inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the

currently named inventors is no longer an inventor of at least one claim remaining in the

application. Any amendment of inventorship must be accompanied by a petition under 37

CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

6.   Any inquiry concerning this communication or earlier communications from the

examiner should be directed to Q. Janice Li whose telephone number is 703-308-7942. The

examiner can normally be reached on 8:30 am - 5 p.m., Monday through Friday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's

supervisor, Deborah J. Reynolds can be reached on 703-305-4051. The fax numbers for the

organization where this application or proceeding is assigned are 703-872-9306 for regular

communications and 703-872-9307 for After Final communications.

Any inquiry of formal matters can be directed to the patent analyst, Dianiece Jacobs,

whose telephone number is (703) 305-3388.

Any inquiry of a general nature or relating to the status of this application or proceeding

should be directed to the receptionist whose telephone number is 703-308-1235. The faxing of

such papers must conform to the notice published in the Official Gazette 1096 OG 30

(November 15, 1989).

Q. Janice Li
Examiner
Art Unit 1632

QJL
February 21, 2003

EXHIBIT B

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
    Chada et al.

Serial No.: 10/017,472

Filed: December 7, 2001

For: METHODS OF TREATMENT
      INVOLVING HUMAN MDA-7

Group Art Unit: 1632

Examiner: Li, Qian J.

Atty. Dkt. No.: INGN:097US

RESPONSE TO RESTRICTION REQUIREMENT DATED FEBRUARY 24, 2003

Commissioner for Patents
Washington, D.C. 20231

Commissioner:

    This paper is submitted in response to the Restriction Requirement dated February 24, 2003 for which the date for response is March 24, 2003.

    It is believed that no fee is due; however, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to this document, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INGN:097US.
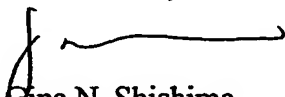
    In response to the restriction requirement that the Examiner imposed, Applicants elect, without traverse, to prosecute claims 1-43, *i.e.*, the Group I claims.

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Claims 1 and 36 are proper linking claims covering both Groups I and II. If claim 1 or claim 36 is found to be allowable, the Group II claims must be rejoined and considered for allowance.

Consistent with this restriction, Applicants will cancel claims 44-67.

The Examiner is invited to contact the undersigned attorney at (512) 536-3081 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,

Gina N. Shishima
Reg. No. 45,104
Attorney for Applicants

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
(512) 474-5201
(512) 536-4598 (facsimile)

Date: March 11, 2003

<u>EXHIBIT C</u>

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/017,472	12/07/2001	Sunil Chada	INGN:097US	5209

7590   09/30/2003

Gina N. Shishima
Fulbright & Jaworski L.L.P.
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Austin, TX   78701

EXAMINER
LI, QIAN J

ART UNIT	PAPER NUMBER
1632	13

DATE MAILED: 09/30/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

RECEIVED 9/30/03
Date(s) Docketed:
Resp. to OAction 3/30/04
(final deadline)

OCT 0 3 2003

Client: INGN:097US
Attorney(s): SLH/GNS
Initials: AM SS

UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/017,472	12/07/2001	Sunil Chada	INGN:097US	5209

7590   09/30/2003

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OCT 0 6 2003

RECEIVED

EXAMINER
LI, QIAN J

ART UNIT	PAPER NUMBER
1632	13

DATE MAILED: 09/30/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

PTO-90C (Rev. 07-01)

	Application No.	Applicant(s)
Office Action Summary	10/017,472	CHADA ET AL.
	Examiner	Art Unit	
	Q. Janice Li	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE _3_ MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on _07 July 2003_ .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) _1-25,32-43 and 68-74_ is/are pending in the application.

    4a) Of the above claim(s) _5,6 and 68-74_ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) _1-4,7-25,32-43_ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on _07 December 2002_ is/are: a)☒ accepted or b)☐ objected to by the Examiner.

    Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

    If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

    a)☐ All b)☐ Some * c)☐ None of:

      1.☐ Certified copies of the priority documents have been received.

      2.☐ Certified copies of the priority documents have been received in Application No. _____ .

      3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

    * See the attached detailed Office action for a list of the certified copies not received.

14)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

    a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1)☒ Notice of References Cited (PTO-892)

2)☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3)☒ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _11_ .

4)☐ Interview Summary (PTO-413) Paper No(s). _____ .

5)☐ Notice of Informal Patent Application (PTO-152)

6)☐ Other: .

DETAILED ACTION

Election/Restrictions

Applicant's election without traverse of Group I, drawn to a method of using a nucleic acid encoding and expressing MDA-7, in Paper No. 9, and supplemental election of species, drawn to a method of treating angiogenesis-dependent cancer using an adenoviral vector expressing fragment 182-206 of SEQ ID No: 2, in Paper No. 12 is acknowledged. In paper #9, applicants indicated that claims 1 and 36 are linking claims of group II and I, upon allowance of group I, claims of group II should be rejoined. In response, Applicants are reminded that the restriction is not issued as linking claim type, because every invention recited in claims 1 and 36 are embraced by groups II and I. Each of the Inventions requires a separate search status and consideration. The inventions are mutually exclusive and independent methods for *in vivo* gene and protein therapies. Therefore, it is <u>maintained</u> that these inventions are distinct due to their divergent subject matter. Further search of these inventions is not co-extensive, as indicated by the separate classifications. The requirement is still deemed proper and is therefore made **FINAL**.

Please note that after a final requirement for restriction, the Applicants, in addition to making any response due on the remainder of the action, may petition the Commissioner to review the requirement. Petition may be deferred until after final action on or allowance of claims to the invention elected, but must be filed not later than

appeal. A petition will not be considered if reconsideration of the requirement was not requested. (See § 1.181.).

Claims 26-31 and 44-67 have been cancelled, claim 6 has been amended, and claims 68-74 are newly submitted. Claims 1-25, 32-43, and 68-74 are pending, however, claims 5, 6, and 68-74 are <u>withdrawn</u> from further consideration by the Examiner, pursuant to 37 CFR 1.142(b), as being drawn to non-elected inventions, there being no allowable generic or linking claim. Claims 1-4, 7-25, 32-43 are under current examination.

Claim Objections

Claims 1, 13, 18-23, 36-38 are objected to because of the following informalities: claims encompass more than one invention as defined in Paper #8, upon election of an invention for examination, said claims should be amended so that they only read upon the elected invention.

Claim 1 is objected to because of the claim recitation, "MDA-7". The abbreviation should be spelled out the first time it appears in the claims.

Claims 16 and 17 are objected to under 37 CFR 1.75(c), as being of improper dependent form for failing to further limit the subject matter of a previous claim. Specifically, claims 16 and 17 are directed to injections performed distally to a disease site, yet depends from a claim directed to local injection (claim 13). Applicant is required to amend the claim(s) to place the claim(s) in proper dependent form, or rewrite the claim(s) in independent form.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-4, 7-25, 32-43 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for *intratumoral injection* of a nucleic acid expressing *full length* MDA-7 polypeptide for treating angiogenesis-dependent cancer, wherein the MDA polypeptide *lacks* a secretory signal, does not reasonably provide enablement for distal or systemic administration of an adenoviral vector expressing *fragments* of MDA-7 polypeptide for treating angiogenesis-dependent tumor, and wherein the MDA-7 polypeptide comprising a secretory signal. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims.

The factors to be considered when determining whether the disclosure satisfies the enablement requirements and whether undue experimentation would be required to make and use the claimed invention are summarized in *In re Wands*, (858 F2d 731, 737, 8 USPQ 2d 1400, 1404, (Fed Cir.1988)). These factors include but are not limited to the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability of the art, the breadth of the claims, and amount of direction provided. The factors most relevant to this rejection are the scope of the claims relative to the state of the art and the levels of the skilled in the art, and whether sufficient

amount of direction or guidance are provided in the specification to enable one of skill in

the art to practice the claimed invention.

Given the broadest reasonable interpretation, the claims encompass treating

cancer with fragments of MDA-7. The specification contemplates that truncated MDA-7

is part of the invention, which encompass fragments ranging from 10 to 206 contiguous

amino acids of SEQ ID No: 2 (Specification, page 13, lines 16-20, for example).

However, neither the specification, nor art of record, teaches a consensus region that is

critical for the function of MDA-7 or the structural correlation of the polypeptide with its

function for inhibiting the growth of tumor cells, and accordingly the specification does

not provide a reasonable guide for those seeking to practice the invention. This is

because the art of protein chemistry is one of the most unpredictable areas of

biotechnology. Although the polynucleotide-coding region determines amino acid

sequence of the protein, it is the conformation of three-dimensional structures that forms

active site, allows the protein to function, and carry out the messages of the genome.

Bowie et al (Science 1990 Mar; 247:1306-10) teach certain position in the sequence are

critical to the three dimensional structure/function relationship and these regions can

tolerate only conservative substitutions or none at all (page 1306, column 2). *Skolnick et*

al (TIBTECH 2000 Jan;18:34-9) teach, "SEQUENCE-BASED METHODS FOR FUNCTION

PREDICTION ARE INADEQUATE BECAUSE OF THE MULTIFUNCTIONAL NATURE OF PROTEINS. HOWEVER,

JUST KNOWING THE STRUCTURE OF THE PROTEIN IS ALSO INSUFFICIENT FOR PREDICTION OF

MULTIPLE FUNCTIONAL SITES" (abstract). They further teach, "KNOWING A PROTEIN'S THREE-

DIMENSIONAL STRUCTURE IS INSUFFICIENT TO DETERMINE ITS FUNCTION" (box 1, page 35). Thus,

one cannot predictably extrapolate the teachings of the specification to the scope of the

claims because the skilled artisan cannot envision the detailed structure of fragments of

SEQ ID No: 2 encompassed by these claims with the function of the fragments.

Moreover, it is unclear exactly what modifications and variations can be tolerated in this

protein and still allows proper tumor-inhibiting function. Determination of the effects of

particular modifications and fragmentations are not predictable until they are actually

made and used, hence resulting in a trial and error situation. *Rudinger* (Peptide

Hormones 1976; June;pages 1-7) teaches the relationship of sequence components

and the peptide hormone function "THE SIGNIFICANCE OF PARTICULAR AMINO ACIDS AND

SEQUENCES FOR DIFFERENT ASPECTS OF BIOLOGICAL ACTIVITY CANNOT BE PREDICTED *A PRIORI* BUT

MUST BE DETERMINED FROM CASE TO CASE BY PAINSTAKING EXPERIMENTAL STUDY." (last

paragraph of text on page 6). The specification fails to provide sufficient teaching for the

fragments of MDA-7, it would have required undue experimentation for the skilled

artisan intending to practice the instant invention.

With respect to the secretory signal, *Su et al* (PNAS 1998;9514400-5, IDS/C65)

teach that the tumor-suppressing effect of mda-7 is associated with chromatin

remodeling via its nucleus translocation from the cytosol, and facilitating the migration of

mda-7 into the nucleus would enhance the selective growth inhibition of malignant but

not normal cells (§ Discussion, page 14404). In view of such teaching, addition of a

secretory signal on MDA-7 polypeptide, would prohibit the nucleus translocation, thus

may abolish the anti-tumor effect of mda-7. In view of such, the invention does not

appear to be enabled in the absence of clarification of the contradictory evidence found in the cited references.

Claims also contemplate administering a (*any*) nucleic acid, naked or in any type of vector, particularly adenoviral vector encoding mda-7 through regional and systemic delivery from a site *distal* from the site of the disease. However, the specification fails to teach how the nucleic acid could reach the target site in a sufficient amount so that a therapeutic effect of tumor killing would achieve. While progress has been made in recent years for gene transfer *in vivo*, vector targeting to desired cells *in vivo* continues to be unpredictable and inefficient as supported by numerous teachings available in the art. For example, *Deonarain* (1998, Expert Opin. Ther. Pat., Vol. 8, pages 53-69) indicate that one of the biggest problems hampering successful gene therapy is the "ABILITY TO TARGET A GENE TO A SIGNIFICANT POPULATION OF CELLS AND EXPRESS IT AT ADEQUATE LEVELS FOR A LONG ENOUGH PERIOD OF TIME" (page 53, first paragraph). Deonarain reference gives high hope to targeted gene delivery, but the discussed strategies are still under investigation, and at the time, they were much less efficient than viral gene delivery (Conclusion).

The claims are drawn to using any naked polynucleotides and vectors. However, whether the recited vectors are suitable for the purpose of the instant invention are unclear. For example, adenoviral vectors are known for their tissue tropism of respiratory epithelial cells, which would be a critical limitation for targeting any angiogenesis-dependent cancer. *Miller et al* (1995, FASEB J., Vol. 9, pages 190-199), acknowledge various vector system available in the art, then teach, "NO SINGLE DELIVERY

SYSTEM IS LIKELY TO BE UNIVERSALLY APPROPRIATE, FOR INSTANCE, THE REQUIREMENTS OF GENE

THERAPY FOR CYSTIC FIBROSIS ARE GREATLY DIFFERENT FROM THOSE OF CANCER" (1st paragraph,

page 190). "ONCE AGAIN, TARGETING AT THE LEVEL OF THE VECTOR HAS NOT YET BEEN

PARTICULARLY WELL DEVELOPED; HENCE, LIPOSOME OR VIRAL-MEDIATED DELIVERY OF THE CFTR

GENE TO AIRWAY EPITHELIAL CELLS OF CF PATIENTS HAS RELIED LARGELY ON THE LOCALIZED

DELIVERY OF THE VECTORS DIRECTLY TO THE AFFECTED TISSUES" (1st paragraph, page 198)

Makrides et al (Protein Exp Pur 1999;17:183-202) teach "THE CHOICE OF AN EXPRESSION

SYSTEM FOR PRODUCTION OF RECOMBINANT PROTEINS DEPENDS ON MANY FACTORS, INCLUDING

CELL GROWTH CHARACTERISTICS, EXPRESSION LEVELS, INTRACELLULAR AND EXTRACELLULAR

EXPRESSION, POSTTRANSLATIONAL MODIFICATIONS AND BIOLOGICAL ACTIVITY OF THE PROTEIN OF

INTEREST, AS WELL AS REGULATORY ISSUES AND ECONOMIC CONSIDERATIONS IN THE PRODUCTION

OF THERAPEUTIC PROTEINS." *Boucher et al* (J Clin Invest 1999 Feb; 103:441-5) review that

host cell resistance to foreign gene is another difficulty for successful in vivo gene

transfer. "DESPITE AN IMPRESSIVE AMOUNT OF RESEARCH IN THIS AREA, THERE IS LITTLE EVIDENCE

TO SUGGEST THAT AN EFFECTIVE GENE-TRANSFER APPROACH FOR THE TREATMENT OF CF LUNG

DISEASE IS IMMINENT. THE INABILITY TO PRODUCE SUCH A THERAPY REFLECTS IN PART THE LEARNING

CURVE WITH RESPECT TO VECTOR TECHNOLOGY AND THE FAILURE TO APPRECIATE THE CAPACITY OF

THE AIRWAY EPITHELIAL CELLS TO DEFEND THEMSELVES AGAINST THE PENETRATION BY MOIETIES,

INCLUDING GENE-THERAPY VECTORS, FROM THE OUTSIDE WORLD." The specification fails to

teach how to overcome the aforementioned difficulties in the art. It would have required

undue experimentation for the skilled artisan intending to practice the instant invention.

Thus, it is evident that at the time of the invention, the gene therapy practitioner,

while acknowledging the significant potential of gene therapy for cancer, still recognized

that such therapy was neither routine nor accepted, and awaited significant

development and guidance for its practice. Therefore, it is incumbent upon applicants to

provide sufficient and enabling teachings within the specification for such therapeutic

regimen. Although the instant specification provides a brief review of a potential

therapeutic use of the claimed method and data from ex vivo and animal studies, it is

not enabled for its full scope because the specification does not disclose the structural-

function relationship of MDA-7 fragments, whether the nucleic acids encompassed by

the claims would function properly in vivo by any means of delivery.

Accordingly, in view of the quantity of experimentation necessary to determine

the parameters for achieving *in vivo* gene expression in selected cells at therapeutic

levels, in particular with any fragment of MDA-7 and any type of nucleic acids, the lack

of direction or guidance provided by the specification as well as the absence of working

examples with regard to targeted *in vivo* gene therapy with fragments of MDA-7

delivered by regional and systemic routes, and the breadth of the claims directed to the

use of numerous fragments, it would have required undue experimentation for one

skilled in the art to make and/or use the claimed invention.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly
claiming the subject matter which the applicant regards as his invention.

Claim 9 is rejected under 35 U.S.C. 112, second paragraph, as being indefinite

for failing to particularly point out and distinctly claim the subject matter which applicant

regards as the invention.

Claim 9 is vague and indefinite because of the unit information is incomplete. The recited "pfu" could be the viral stock solution of "pfu per mL" or the infected cell concentration, "pfu per cell", it is unclear which one the applicants intend to claim, and thus the metes and bounds of the claim are uncertain.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claims 1-4, 7, 8, 10-15, 24, 25, 35, 36, 42, 43 are rejected under 35 U.S.C. 102(e) as being anticipated by *Fisher* (US 6,355,622).

Fisher teaches a method of inhibiting angiogenesis dependent cancer in a subject suffering from cancer comprising intratumoral administering to nude mice bearing human cervical carcinoma cells replication deficient adenoviral vector encoding mda-7 gene (AA 1-206 of SEQ ID No. 2) three times a week for 4 weeks, the well-established tumors were growth inhibited in the treated mice compared to the control group (column 14, lines 35-67), wherein the expression of mda-7 was driven by a CMV promoter (column 13, line 56). *Fisher* also teaches that the nucleic acid could be embedded in liposomes and introduced into the cell (column 3, line 67, lipid

composition). *Fisher* teaches that ectopic expression of mda-7 inhibits the growth of

tumor cells and may provide therapeutic benefit for the treatment of human cancer

(column 14, lines 62-65). Therefore, *Fisher* anticipates the instant claims.

Claims 1-4, 7-25, 35-43 are provisionally rejected under 35 U.S.C. 102(e) as

being anticipated by copending Application No. 09/615,154 which has a common

inventor with the instant application. Based upon the earlier effective U.S. filing date of

the copending application, it would constitute prior art under 35 U.S.C. 102(e), if

published under 35 U.S.C. 122(b) or patented. This provisional rejection under 35

U.S.C. 102(e) is based upon a presumption of future publication or patenting of the

copending application.

Claims of instant application and the cited application are each drawn to a

method of treating a tumor patient comprising administering a viral vector expressing a

mda-7 polypeptide or fragment 182-206 of SEQ ID No:2 combined with conventional

chemotherapy, surgery, and radiation therapy. Considerable overlap in the scope of the

claims is present. Therefore, the inventions as claimed are co-extensive.

This provisional rejection under 35 U.S.C. 102(e) might be overcome either by a

showing under 37 CFR 1.132 that any invention disclosed but not claimed in the

copending application was derived from the inventor of this application and is thus not

the invention "by another," or by an appropriate showing under 37 CFR 1.131. This

rejection may not be overcome by the filing of a terminal disclaimer. See *In re Bartfeld*,

925 F.2d 1450, 17 USPQ2d 1885 (Fed. Cir. 1991).

Claims 1-4, 7-25, 35-43 are rejected under 35 U.S.C. 102(f) because the

applicant did not invent the claimed subject matter. U.S. patent application 09/615,154

has a different inventive entity, yet the disclosure anticipates the instantly claimed

invention. It is unclear as to who is the real inventor. Appropriate clarification is required.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all

obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1, 7-9, 20-23, 36-41 are rejected under 35 U.S.C. 103(a) as being

unpatentable over *Roth et al* (US 6,069,134), in view of *Fisher* (US 6,355,622).

Roth et al teach a method comprising administering DNA damaging agent

combined with adenoviral vector expressing a tumor suppressor (particularly p53,

abstract), together with conventional chemotherapy and surgery for the treatment of

cancer (column 3, lines 20-48). *Roth et al* teach that the DNA damaging agents include

gamma-irradiation, x-rays and UV-irradiation, for example; and the chemotherapeutic

agents include 5-fluorouracil (column 4, lines 57-67). *Roth et al* also teach that the

adenoviral stock was administered at a m.o.i. of 10^8 pfu/ml (column 12, line 1). *Roth et*

al do not teach that the tumor suppressor is MDA-7.

Fisher teaches that using adenovirus encoding MDA-7 as the tumor suppressor for treatment of cancer, and administering the vector to tumor cells *in vitro* at moi of 10^2pfu/cell, but does not specify the dosage for *in vivo* administration (column 14, line 22). *Fisher* teaches that ectopic expression of mda-7 inhibit the growth of tumor cells and may provide therapeutic benefit for the treatment of human cancer in general, but did not discuss the details of such therapy (column 14, lines 62-65).

Claims 20-23 and 37-41 are limitations for the timing of the combination therapy, neither Roth et al nor Fisher discuss the details. However, given the levels of the ordinary skilled in the art, these limitations would fall within the bounds of the optimization for a proper therapeutic regimen.

Accordingly, it would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the methods taught by *Roth et al* by simply substituting the p53 with mda-7 as taught by *Fisher et al* and administering the mda-7 either prior or after the conventional therapy at a dosage sufficient for tumor cell killing with a reasonable expectation of success. The ordinary skilled artisan would have been motivated to modify the claimed invention because the combined therapy would maximize the tumor-treating effect by any individual therapy alone. Thus, the claimed invention as a whole was *prima facie* obvious in the absence of evidence to the contrary.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the

unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970);and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 1-4, 7-25, 32, and 35-43 are provisionally rejected under the judicially

created doctrine of obviousness-type double patenting as being unpatentable over

claims 91-116,125-154, 159-174 of copending U.S. Patent Application No. 09/615,154.

Although the conflicting claims are not identical, they are not patentably distinct

from each other because the present application and the claims of the cited patent are

each drawn to a method for treating a tumor patient comprising administering a viral

vector expressing a mda-7 polypeptide combined with conventional chemotherapy,

surgery, and radiation therapy. Considerable overlap in the scope of the claims is also

present.

Accordingly, the claimed processes in the copending and the present application

are obvious variants. Therefore, the inventions as claimed are co-extensive.

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the

examiner should be directed to Q. Janice Li whose telephone number is 703-308-7942.

The examiner can normally be reached on 8:30 am - 5 p.m., Monday through Friday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's

supervisor, Deborah J. Reynolds can be reached on 703-305-4051. The fax numbers

for the organization where this application or proceeding is assigned are 703-872-9306

for regular communications and 703-872-9307 for After Final communications.

Any inquiry of formal matters can be directed to the patent analyst, Dianiece

Jacobs, whose telephone number is (703) 305-3388.

Any inquiry of a general nature or relating to the status of this application or

proceeding should be directed to the receptionist whose telephone number is 703-308-

1235. The faxing of such papers must conform to the notice published in the Official

Gazette 1096 OG 30 (November 15, 1989).

Q. Janice Li
Patent Examiner
Art Unit 1632

September 22, 2003

Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/017,472
List of Patents and Publications for Applicant's INFORMATION DISCLOSURE STATEMENT	Applicant Sunil Chada *et al.*	
(Use several sheets if necessary)	Filing Date: December 7, 2001	Group: 1645
U.S. Patent Documents *See Page 1*	Foreign Patent Documents *See Page 2*	Other Art *See Page 2*

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U.S. Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Name	Class	Sub Class	Filing Date of App.
X	A1	4,682,195	7-21-87	Yilmaz	357	23.4	9-30-85
✓	A2	4,683,202	7-28-87	Mullis	435	91	10-25-85
	A3	4,797,368	1-10-89	Carter *et al.*	435	320	3-15-85
	A4	5,139,941	8-18-92	Muzyczka *et al.*	435	172.3	10-25-91
	A5	5,399,363	3-21-95	Liversidge *et al.*	424	490	7-1-92
	A6	5,466,468	11-14-95	Schneider *et al.*	424	450	10-28-94
	A7	5,543,158	8-6-96	Gref *et al.*	424	501	7-23-93
	A8	5,633,016	5-27-97	Johnson	424	649	5-1-95
	A9	5,641,515	6-24-97	Ramtoola	424	189	6-7-95
	A10	5,645,897	7-8-97	Andra	427	526	1-18-95
	A11	5,705,629	1-6-98	Bhongle	536	25.34	10-20-95
	A12	5,739,169	4-14-98	Ocain *et al.*	514	658	5-31-96
	A13	5,798,339	8-25-98	Brandes	514	34	6-28-93
	A14	5,801,005	9-1-98	Cheever *et al.*	435	7.24	3-31-95
	A15	5,824,311	10-20-98	Greene *et al.*	424	138.1	11-30-94
	A16	5,824,348	10-20-98	Fujiu *et al.*	425	120	1-16-97
	A17	5,830,880	11-3-98	Sedlacek *et al.*	514	44	4-18-97
	A18	5,846,225	12-8-98	Rosengart *et al.*	604	115	2-19-97
✗	A19	5,846,233	12-8-98	Lilley *et al.*	604	414	1-9-97
✓	A20	5,846,945	12-8-98	McCormick	514	44	6-7-95

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EXAMINER:	DATE CONSIDERED: 9/9/03

EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

INFORMATION DISCLOSURE STATEMENT — PTO-1449 (MODIFIED)

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Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/017,472
List of Patents and Publications for Applicant's INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	Applicant Sunil Chada *et al.*	
	Filing Date: December 7, 2001	Group: 1645

U.S. Patent Documents *See Page 1*	Foreign Patent Documents *See Page 2*	Other Art *See Page 2*

Foreign Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Country	Class	Sub Class	Translation Yes/No
	B1	266032	5-4-88	Europe			
	B2	WO 00/05356	2-3-00	PCT			
	B3	WO 00/26368	5-11-00	PCT			
	B4	WO 95/11986	5-4-95	PCT			
	B5	WO 98/07408	2-26-98	PCT			
	B6	WO 98/28425	7-2-98	PCT			

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C1	Anderson, "Human gene therapy," *Nature*, 392:25-30, 1998.
	C2	Austin-Ward and Villaseca, "Gene therapy and its applications," *Rev. Med. Chil.*, 126:838-845, 1998. Article in Spanish, Abstract included.
	C3	Blumberg *et al.*, "Inyrtlrukin 20: discovery, receptor identification, and role in epidermal function," *Cell* 104:9-19, 2001.
	C4	Bonavida *et al.*, "Selectivity of TRAIL-mediated apoptosis of cancer cells and synergy with drugs: The trail to non-toxic cancer therapeutics," *Int J Oncol*, 15:793-802, 1999.
	C5	Bukowski *et al.*, "Signal transducton abnormalities in T lymphyocytes from patients with advanced renal carcinoma: Clinical relevance and effects of cytokine therapy," *Clin. Cancer Res.*, 4(10):2337-2347, 1998.
	C6	Chinnaiyan *et al.*, "Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy," *Proc Nat'l Acad Sci USA.* 97:1754-1759, 2000.
	C7	Christodoulides *et al.*, "Immunization with recombinant class 1 outer-membrane protein from *neisseria meningitidis*: influence of liposomes and adjuvants on antibody avidity, recognition of native protein and the induction of a bactericidal immune response against meningococci," *Microbiology*, 144(Pt 11):3027-3037, 1998.
	C8	Crystal, "Transfer of genes to humans: early lessons and obstacles," *Science*, 270:404-409, 1995.

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EXAMINER:	DATE CONSIDERED: 9/9/03

EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

INFORMATION DISCLOSURE STATEMENT — PTO-1449 (MODIFIED)

Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/017,472
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	Filing Date: December 7, 2001	Group: 1645

U.S. Patent Documents See Page 1	Foreign Patent Documents See Page 2	Other Art See Page 2

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C9	Davidson et al., "Intralesional cytokine therapy in cancer: A pilot study of GM-CSF infusion in mesothelioma," *J. Immunother.*, 21:389-398, 1998.
	C10	De Waal Malefyt et al., "Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes," *J. Exp. Med.* 174:1209-1220, 1991.
	C11	Dragovich et al., "Signal transduction pathways that regulate cell survival and cell death," *Oncogene*, 17:3207-3213, 1998
	C12	Dumoutier et al., "Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor," *Proc. Natl. Acad. Sci. USA.* 97:10144-10149, 2000.
	C13	Ekmekcioglu et al., "Differential increase of Fas ligand expression on metastatic and thin or thick primary melanoma cells compared with interleukin-10," *Melanoma Research* 9:261-272, 1999.
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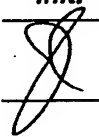| Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/017,472
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	Filing Date: December 7, 2001	Group: 1645

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Filing Date: December 7, 2001	Group: 1645

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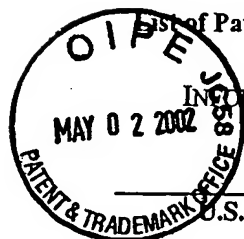| Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/017,472
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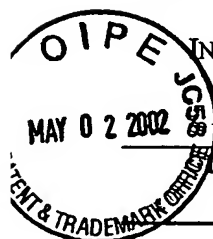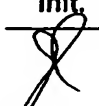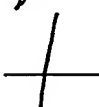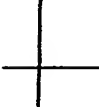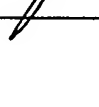
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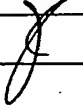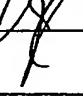
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		Examiner	Art Unit	
		Q. Janice Li	1632	Page 1 of 2

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10/017,472	CHADA ET AL.
Examiner	Art Unit	Page 2 of 2
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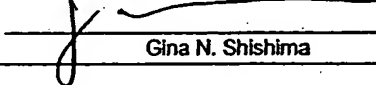
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EXHIBIT D

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
    Chada et al.   Group Art Unit: 1632

Serial No.: 10/017,472   Examiner: Li, Qian J.

Filed: December 7, 2001   Atty. Dkt. No.: INGN:097US

For: METHODS OF TREATMENT
    INVOLVING HUMAN MDA-7

I. AMENDMENT; AND II. RESPONSE TO OFFICE ACTION DATED SEPTEMBER 30, 2003

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

This paper is submitted in response to the Office Action dated September 30, 2003 for

which the three-month date for response was December 30, 2003.

A request for a one-month extension of time to respond is included herewith along with

the required fee. This extension will bring the due date to January 30, 2004, which is within the

six-month statutory period. Should such request or fee be deficient or absent, consider this

paragraph such a request and authorization to withdraw the appropriate fee under 37 C.F.R.

§§ 1.16 to 1.21 from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INGN:097US.

Amendments to the claims are reflected in the listing of claims, which begins on page 3 of this paper.

Remarks/Arguments in response to the Office Action begin on page 9 of this paper.

I. AMENDMENT

Listing of Claims

1. (Currently amended) A method of inhibiting angiogenesis in a <u>human</u> patient in need of such treatment comprising administering to the patient <u>an effective amount of</u> a human melanoma differentiation antigen-7 (MDA-7) polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells <u>to inhibit angiogenesis,</u> ~~whereby the MDA-7 polypeptide inhibits angiogenesis in the patient~~.

2. (Original) The method of claim 1, wherein said patient exhibits an angiogenesis-related disease.

3. (Original) The method of claim 2, wherein the angiogenesis-related disease is further defined as angiogenesis-dependent cancer, a benign tumor, rheumatoid arthritis, psoriasis, an ocular angiogenic disease, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, a telangiectasia, hemophiliac joint, angiofibroma, wound granulation, cat scratch disease, an ulcer, an intestinal adhesion, atherosclerosis, scleroderma, or a hypertrophic scar.

4. (Original) The method of claim 3, wherein angiogenesis-dependent cancer is further defined as a solid tumor, leukemia, or a tumor metastasis.

5. (Withdrawn) The method of claim 3, wherein the benign tumor is further defined as a hemangioma, a neuroma, a neurofibroma, a trachoma, uterine fibroid, hamartoma, teratoma, or a pyogenic granuloma.

6. (Withdrawn) The method of claim 3, wherein the ocular angiogenic disease is further defined as diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, or Rubeosis.

7.   (Original) The method of claim 1, wherein the nucleic acid is an expression vector.

8.   (Original) The method of claim 7, wherein the expression vector is a viral vector.

9.   (Original) The method of claim 8, wherein the viral vector is administered at between 10^3 and 10^{13} pfu.

10.   (Original) The method of claim 8, wherein said viral vector is an adenoviral vector, a retroviral vector, a vaccinia viral vector, an adeno-associated viral vector, a polyoma viral vector, or a herpesviral vector.

11.   (Original) The method of claim 8, wherein said viral vector is an adenoviral vector.

12.   (Original) The method of claim 1, wherein said nucleic acid further comprises a CMV IE, dectin-1, dectin-2, human CD11c, F4/80, SM22 or MHC class II promoter.

13.   (Original) The method of claim 1, wherein the MDA-7 polypeptide or nucleic acid is administered to the patient by direct injection into an area in need of inhibition of angiogenesis.

14.   (Original) The method of claim 13, wherein the patient is administered multiple injections.

15.   (Currently amended) The method of claim [13] 1, wherein the injection is performed locally to a disease site.

16.   (Currently amended) The method of claim [13] 1, wherein the injection is performed regionally to a disease site.

17.   (Currently amended) The method of claim [13] 1, wherein the injection is performed distally to a disease site.

18.　(Original)　The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered to the patient by continuous infusion.

19.　(Original)　The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered to the patient by intravenous injection.

20.　(Original)　The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered prior to or after surgery.

21.　(Original)　The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered before chemotherapy, immunotherapy, or radiotherapy.

22.　(Original)　The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered during chemotherapy, immunotherapy, or radiotherapy.

23.　(Original)　The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered after chemotherapy, immunotherapy, or radiotherapy.

24.　(Original)　The method of claim 1, wherein the patient is a human.

25.　(Original)　The method of claim 1, wherein the MDA polypeptide comprises amino acids from 1 to 206 of SEQ ID NO:2.

26.-31.　(Cancelled)

32.　(Original)　The method of claim 1, wherein the MDA polypeptide comprises amino acids from 182 to 206 of SEQ ID NO:2.

33.　(Original)　The method of claim 1, wherein the MDA polypeptide comprises a secretory signal.

34. (Original) The method of claim 33, wherein the secretory signal is further defined as a positively charged N-terminal region in combination with a hydrophobic core.

35. (Original) The method of claim 1, wherein the patient is a cancer patient.

36. (Currently amended) A method of inhibiting endothelial cell differentiation in a human patient comprising administering to the patient an effective amount of a human MDA-7 polypeptide or a nucleic acid molecule expressing the human MDA-7 polypeptide.

37. (Original) The method of claim 36, wherein a chemotherapeutic agent is administered prior to administration of the MDA-7 polypeptide or the nucleic acid molecule.

38. (Original) The method of claim 36 wherein a chemotherapeutic agent is administered after administration of the MDA-7 polypeptide or the nucleic acid molecule.

39. (Original) The method of claim 36, wherein the chemotherapeutic agent is a DNA damaging agent.

40. (Original) The method of claim 39, wherein the DNA damaging agent is gamma-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), or hydrogen peroxide.

41. (Original) The method of claim 38, wherein the chemotherapeutic agent is a cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin, methotrexate, or analog or derivative variant thereof.

42.　(Original)　The method of claim 36, wherein the nucleic acid is comprised within a viral vector.

43.　(Original)　The method of claim 36, wherein the nucleic acid is comprised in a lipid composition.

68.　(Withdrawn)　The method of claim 32, wherein the MDA polypeptide comprises amino acids from 175 to 206 of SEQ ID NO:2.

69.　(Withdrawn)　The method of claim 68, wherein the MDA polypeptide comprises amino acids from 150 to 206 of SEQ ID NO:2.

70.　(Withdrawn)　The method of claim 69, wherein the MDA polypeptide comprises amino acids from 125 to 206 of SEQ ID NO:2.

71.　(Withdrawn)　The method of claim 70, wherein the MDA polypeptide comprises amino acids from about 100 to about 206 of SEQ ID NO:2.

72.　(Withdrawn)　The method of claim 71, wherein the MDA polypeptide comprises amino acids from 75 to 206 of SEQ ID NO:1.

73.　(Withdrawn)　The method of claim 72, wherein the MDA polypeptide comprises amino acids from 49 to 206 of SEQ ID NO:2.

74.　(Withdrawn)　The method of claim 73, wherein the MDA polypeptide comprises amino acids from 1 to 206 of SEQ ID NO:2.

75.　(New)　The method of claim 8, wherein 10^{10} to 10^{13} viral particles are administered.

76.　(New)　The method of claim 75, wherein 10^{11} to 10^{12} viral particles are administered.

77. (New) The method of claim 3, wherein the angiogenesis-dependent cancer is a hepatocarcinoma, retinoblastoma, astrocytoma, leukemia, neuroblastoma, mesothelioma, or non-small cell lung, small-cell lung, lung, head, neck, pancreatic, prostate, renal, bone, testicular, ovarian, cervical, gastrointestinal, lymphoma, brain, colon or bladder cancer.

II. RESPONSE TO OFFICE ACTION

A. Status of the Claims

Claims 1-25, 32-43, and 68-74 were pending prior to the Office Action dated September 30, 2003. Claims 1, 15-17, and 36 have been amended. Support for the amendments may be found throughout the specification, for example, at page 10, lines 7-10 and page 22, lines 21-25 and in the originally filed claims, such as claim 36. Claims 75-77 have been added. Support for these claims can be found in the specification, for example, at page 56, lines 27-28 and at page 9, lines 9-13, 18-20. No new matter has been added.

The withdrawal of claims 68-74 from consideration is inappropriate. Claim 32 contains the elected species and added claims 68-74 depend from claim 32 and further limit the elected species.

B. Claim Objections

1. Amendment of Claims Due to Election

The Action objects to claims 1, 13, 18-23, and 36-38 because they encompass more than one invention as defined in the restriction requirement (Paper No. 8). The Action contends the claims should be amended so that they read only upon the elected invention. Applicants respectfully traverse this objection.

The MPEP states, "The linking claims must be examined with the invention elected, and should any linking claim be allowed, the restriction requirement must be withdrawn." MPEP 809. Linking claims include "genus claims linking species claims." MPEP 809.03. Claims 1 and 36 are linking claims.

Applicants respectfully request that the examiner provide the authority or citation that requires an applicant to *amend* claims based merely on an election of invention.

2. Recitation of "MDA-7"

The Action objects to claim 1 because of its recitation of "MDA-7" and requires that the abbreviation be spelled out. Claim 1 now recites "Melanoma Differentiation Antigen-7 (MDA-7)."

3. Claims 16 and 17 Are Amended

The Action objected to claims 16 and 17 as being improperly dependent. Claim 16 has been amended to depend from claim 1 instead of claim 13. Similarly, claim 17 has been amended to depend from claim 1 as well.

C. Claims 1-4, 7-25, and 32-43 Are Enabled

The Action rejects claim 1-4, 7-25, and 32-43 under 35 U.S.C. §112, first paragraph, because the specification allegedly does not enable the claimed invention for the following aspects: (1) MDA-7 polypeptide fragments; (2) addition of a secretory signal on an MDA-7 polypeptide; (3) targeting of a nucleic acid to a target site to effect tumor killing. Applicants respectfully traverse this rejection.

1. MDA-7 Fragments Are Enabled

The Action contends that neither the specification, nor art of record, teaches a consensus region that is critical for the function of MDA-7 or the structural correlation of the polypeptide with its function for inhibiting the growth of tumor cells. It also contends that the art of protein chemistry is "one of the most unpredictable areas of biotechnology." It cites the references of Bowie *et al.* as teaching that certain positions in a protein sequence are critical to the three-dimensional structure/function relationship and that these regions can tolerate only conservative substitutions or none at all (page 1306, column 2). The Action also cites the reference of Skolnick *et al.* to support its conclusion that one cannot predictably extrapolate the teaching of the specification to the scope of the claims because the skilled artisan cannot envision the

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detailed structure of fragments of SEQ ID NO:2 encompassed by these claims with the function of the fragments. Moreover, it states that determination of the effects of particular modifications and fragmentations are not predictable until they are actually made, citing the reference of Rudinger. For these reasons, the Action concludes that it would have required undue experimentation to practice the invention.

The Action's contention that the specification fails to provide a sufficient teaching for fragments of MDA-7 lacks merit. A closer look at the cited references does not support the Action's conclusion.

For example, the Action's citation to the Skolnick reference is taken out of context with respect to the issue at hand. The Action cites Skolnick for stating 1) "Sequence-based methods for function prediction are inadequate because of the multifunctional nature. However just knowing the structure of the protein is also insufficient for prediction of multiple functional sites" (abstract)"; and 2) "Knowing a protein's three-dimensional structure is insufficient to determine its function." The entire Skolnick paper is focused on the issue of *predicting* what a protein's function *might be* when *only* sequence information is available, such as in the context of genome sequencing-type projects, where cDNA sequences are obtained. This is reflected by the title of the reference, "From genes to protein structure and function: novel applications of computational approaches in the genomic era." The Skolnick reference might be relevant if Applicants were claiming a cDNA sequence for which no utility had been established. However, this reference is not relevant to the claimed invention because a function for MDA-7 is *already* provided and this is recited in the claims.

The Action contends that "Determination of the effects of particular modifications and fragmentations are not predictable until they are actually made and used, hence resulting in a trial

and error situation." Action at page 6. However, the standard for enablement is not the need for "trial and error." The test of enablement is whether the experimentation needed to practice the invention is undue. MPEP § 2164.01 (citing *Mineral Separation v. Hyde*, 242 U.S. 261, 270 (1916)). In fact, satisfaction of the enablement requirement is not precluded by the necessity of some experimentation. *See Atlas Powder Co. v. E.I. duPont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 U.S.P.Q. 409 (Fed. Cir. 1984). Therefore, even if trial and error were required to practice the invention, the Action has still not met its burden of showing that this is tantamount to requiring "undue experimentation."

The Action relies upon the reference of Rudinger to indicate that "painstaking experimental study" is required to predict the significance of particular amino acids and sequences for different aspects of biological activity. However, this reference is irrelevant to the present invention because it was published in 1976, *almost 25* years before the current application was filed. Surely this reference does not reflect the state of the art at the time the application was filed. Particularly notable is the fact that in the last 25 years, recombinant DNA technology has made something that was extremely difficult—requiring perhaps "painstaking experimental study"—25 years ago, such as cloning a gene, a trivial pursuit, as is demonstrated by the completion of the Human Genome Project in the last two years.

In fact, a skilled artisan could readily prepare fragments covered by the claims and test them for function. The specification provides the cDNA sequence for MDA-7 and teaches, for example, that fragments can be generated recombinantly. Specification at pages 35-44.

Applicants respectfully note that the PTO is required, when examining a patent application, to assume that the specification complies with §112 unless it has "acceptable evidence or reasoning" to suggest otherwise. *In re Marzocchi*, 439 F.2d 220, 223-24, 169 USPQ

367, 369-370 (CCPA. 1971). Thus, the PTO must provide reasons supported by the record as a whole what the specification is not enabling. *Application of Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219-220 (CCPA 1979). Then and only then does the burden shift to the Applicants to prove that one of ordinary skill in the art could have practiced the claimed invention without undue experimentation. *In re Strahilevitz*, 668 F.2d. 1229, 1232, 212 USPQ 561, 563-64 (CCPA 1982). In this case, the cited references regarding protein sequence and structure do not shift the burden to the Applicants.

Moreover, Applicants provide additional evidence regarding an MDA-7 fragment. The Declaration of Sunil Chada ("Declaration") (Appendix A) indicates that an MDA-7 polypeptide lacking the first 48 amino acids of the full-length sequence induced cell killing in melanoma cells. Declaration at ¶ 6. Moreover, an MDA-7 polypeptide lacking the first 48 amino acids but containing a sequence targeting it to the endoplasmic reticulum suppressed the growth of prostate cancer cells (PC3 cells) and human non-small cell lung carcinoma cells (H1299 cells). Declaration at ¶ 7. Therefore, in view of the foregoing arguments, Applicants respectfully request this ground for the rejection be withdrawn.

2.   Secretory Signal with MDA-7 Is Enabled

The Action contends that the reference of Su *et al.* teaches that the tumor suppressing effect of MDA-7 is associated with chromatin remodeling via its nucleus translocation from the cytosol and facilitating the migration of MDA-7 into the nucleus would enhance the selective growth inhibition of malignant but not normal cells. Therefore, the Action contends that the addition of a secretory signal on an MDA-7 polypeptide would prohibit the nucleus translocation and thus abolish the anti-tumor effect of MDA-7.

First, the Su reference cited by the Action does not provide data to indicate that addition of a secretory signal would abolish the anti-tumor effects of MDA-7; it merely speculates in the

Discussion section that facilitating migration of MDA-7 into the nucleus may enhance growth inhibition.

Moreover, the Declaration of Sunil Chada indicates that the first 48 amino acids of the full-length sequence may be cleaved to yield a secreted form of the protein. The present specification indicates that there is a putative secretory signal in the first 46 amino acids of the protein. Specification at page 26, lines 1-9. Furthermore, the Declaration indicates that different forms of MDA-7 were evaluated in prostate cancer cells and human non-small cell lung carcinoma cells. An MDA-7 lacking its own secretion signal but containing a signal targeting it to the endoplasmic reticulum (ER version) showed growth suppression in those cells, as did the full-length MDA-7. However, an MDA-7 targeted to the nucleus or an MDA-7 targeted to the cytoplasm did not. Also, higher levels of apoptosis were observed in cells transfected with full-length and ER versions of MDA-7, compared to the cytoplasmic or nuclear versions of MDA-7.

Therefore, there should not be any issues regarding whether an MDA-7 polypeptide with a signal sequence attached to it can achieve the anti-tumor effect of MDA-7.

3.   Administration of MDA-7 Encoding Nucleic Acids Are Enabled

The Action generally contends that while progress has been made in recent years for gene transfer *in vivo*, targeting of naked nucleic acid or any vector to desired cells *in vivo* continues to be unpredictable and inefficient. While difficult to discern clearly, the Action seems to be making three points: 1) gene therapy is unpredictable; 2) targeting of a nucleic acid that is not a viral vector (nonviral vector-nucleic acid) is problematic; and 3) targeting of viral vectors, for example adenovirus, may be problematic.

i)   Gene therapy

The Action contends that the gene therapy practitioner, while acknowledging the significant potential of gene therapy for cancer, still recognizes that such therapy was neither

routine nor accepted and await significant development and guidance for its practice. It cites the references of Miller *et al.*, Makrides *et al.*, and Boucher *et al.* to allegedly support its contention.

Once again, a closer examination of the cited references reveals that they do not support the Action's conclusions and also, there is evidence that indicates gene therapy can be practiced according to the specification and knowledge of the skilled artisan.

The Action cites the reference of Miller as saying, "No single delivery system is likely to be universally appropriate, for instance, the requirements of gene therapy for cystic fibrosis are greatly different from those of cancer." Action at page 8, citing page 190 of Miller. By its own admission, the Action renders the next citation to Miller and the citation to Boucher irrelevant because they both involve statements relating to the treatment of cystic fibrosis, while the present invention is related to inhibiting angiogenesis.

As for the reliance on the reference of Makrides, this reference merely states that "the choice of an expression system for production of recombinant proteins depends on many factors...." However, it is not clear how this statement indicates that undue experimentation would be required to practice the invention. Moreover, this reference says nothing about the ability to express MDA-7 or any limitations there might be with its expression.

In fact, there is evidence to support the contention that the claims are enabled. In addition to the data regarding a therapeutic effect from administration of Ad-mda7 in the specification (Examples 1, 4, 6, 9, 10 and 11), there is information relating to the administration of an MDA-7-encoding plasmid in a DOTAP:cholesterol liposome to a nude mouse. In the Declaration of Sunil Chada, he sets forth that nude mice with tumors exhibited reduced tumor growth and reduced levels of CD31 staining after treatment with the DOTAP:Chol-*mda-7*

complex. Declaration at ¶ 9. A reduction in levels of CD31 staining is indicative of reduced vascularization, *i.e.*, inhibition of angiogenesis.

ii) Nonviral-vector nucleic acids

To support its argument that gene therapy using a nonviral vector-nucleic acid, the Action refers to the reference of Deonarain. It cites Deonarain as stating that one of the biggest problems hampering successful gene therapy is the "ability to target a gene to a significant population of cells and express it at adequate levels for a long enough period of time." Action at page 7. The Action also says that the Deonarain reference gives high hope to targeted gene delivery, but that the strategies it discusses are still under investigation and that the reference concludes they were much less efficient than viral gene delivery.

However, there are several reasons the Deonarain reference does not support the broad conclusion that gene therapy with nonviral-vector nucleic acids is unpredictable and inefficient.

The Action's quotation from the first line of the abstract regarding "one of the main obstacles" to fulfilling the promise of gene therapy is taken out of context, because the reference goes on to say that "Viral methods of gene delivery have been studied for a number of years and are effective vectors for gene transfer." The Deonarain reference goes on to say that alternative methods are being explored because of issues relating to mutagenesis, side effects and toxicity—not targeting and expression levels.

Also, the conclusion that the Action cites from the Deonarain reference regarding targeted gene delivery being less efficient than viral gene delivery is followed by the statement, "However, under optimal conditions, enough gene product may be produced to give a therapeutic benefit (*e.g.*, suppress a phenotype of destroy a tumour)." Therefore, the use of the

specific nonviral vector nucleic acid delivery method discussed in the article is not plagued with as many problems as the Action contends.

Furthermore, this reference concerns specifically one type of nonviral vector nucleic acid delivery—"ligand-targeted receptor mediated vectors for gene delivery"—as the title indicates. There are other types of nonviral vector technology, which is not discussed by the Action at all. Therefore, even if one particular type of gene therapy is still undergoing experimentation and improvement, that does not mean that the instant claims reciting a "nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells" is not enabled.

Moreover, the concern regarding targeting and sustained expression of a gene may be less significant for a gene such as *mda-7*. As the specification indicates, MDA-7 induces apoptosis, and it selectively induces apoptosis in cancer cells, as opposed to normal cells. Specification at page 75. That MDA-7 induces apoptosis in a cell means expression of MDA-7 does not need to be sustained because once it enters the cell and induces apoptosis, that cell is no longer around. Additionally, because MDA-7 selectively induces apoptosis in cancer cells, targeting and sustained expression of MDA-7 are not the issues that they might be for many other gene therapies. Applicants contend that the Action does not raise provide credible reasons supported by the record for its contentions that undue experimentation would be required to practice the invention because it concerns gene therapy with nonviral vector-nucleic acids.

iii)   Viral vectors

The Action also contends that it is not clear whether the recited vectors are suitable for the purpose of the instant invention. The Action contends that adenoviral vectors, for example, are known for their tissue tropism of respiratory epithelial cells, which would be a critical limitation for targeting any angiogenesis-dependent cancer.

First, the Action does not cite a reference or provide a declaration or affidavit to support this contention. Furthermore, the literature is replete with example of adenovirus infecting a variety of cell types, in addition to respiratory epithelial cells. In fact, the specification of the instant application shows that adenovirus infected breast cancer cells (Example 4), in addition to lung cancer cells (Example 10). Furthermore, the evidence cited above regarding clinical trials of tumor suppressors provides additional evidence that adenovirus can be used as a gene therapy vector, and that it is not limited to respiratory epithelial cells.

D.   Claim 9 is Definite

The Action rejects claim 9 under 35 U.S.C. §112, second paragraph, as being indefinite for its recitation of "pfu." A random search in PubMed for articles published around the time the priority application for this application was filed reveals that "pfu" is used in the literature. Copies of two Journal of Virology papers are provided as examples to show that "pfu" is an appropriate unit dose (See abstracts) (Appendix B).

E.   Claims Are Not Anticipated

       1.   Claims 1-4, 7, 8, 10-15, 24, 25, 35, 36, 42, and 43 Are Not Anticipated by Fisher

The Action rejects claims 1-4, 7, 8, 10-15, 24, 25, 35, 36, 42, and 43 under 35 U.S.C. §102(e) as being anticipated by Fisher (U. S. Patent No. 6, 355,622). Fisher is alleged to teach a method of inhibiting an angiogenesis-dependent cancer in a subject suffering from cancer comprising intratumoral administration of a replication-deficient adenoviral vector encoding the MDA-7 gene (amino acids 1-206 of SEQ ID NO:2) to nude mice bearing human cervical carcinoma cells. The Action contends that Fisher also teaches that the nucleic acid could be imbedded in liposomes introduced into the cell. Finally, it concludes that ectopic expression of MDA-7 inhibits the growth of tumor cells and may provide therapeutic benefit for the treatment

of human cancer, and as such, anticipates the instant claims. Applicants respectfully traverse this rejection.

The Federal Circuit case of *Kalman v. Kimberly-Clark Corp.*, 713 F.2d 760 (Fed. Cir. 1983) states that *identity of invention* is required for anticipation. *Each element* of the claim in issue must be found in a single prior art reference. The claims recite:

> A method of inhibiting angiogenesis in a human patient in need of such treatment comprising administering to the patient an effective amount of a human melanoma differentiation antigen-7 (MDA-7) polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis.

The Fisher patent, however, does not even mention angiogenesis or inhibition of angiogenesis. Accordingly, it does not anticipate the claimed invention. Applicants respectfully request this rejection be withdrawn.

2.   Provisional Rejection Under 35 U.S.C. §102(e) of Claims 1-4, 7-25, and 35-43

The Action provisionally rejects 1-4, 7-25, and 35-43 under 35 U.S.C. §102(e) as being anticipated by copending application number 09/615,154, which has a common inventor with the instant application.

Because this rejection is provisional, Applicants will address this rejection, if necessary, once that application or the current application becomes otherwise allowable.

F.   Claims 1, 7-9, 20-23, and 36-41 Are Not Obvious Over Roth *et al.* in View of Fisher

The Action rejects claims 1, 7-9, 20-23, and 36-41 under 35 U.S.C. §103(a) as being unpatentable over Roth *et al.* (U. S. Patent No. 6,069,134) in view of Fisher (U. S. Patent No. 6,355,622). It alleges that Roth teaches a method of administering a DNA damaging agent with an adenoviral vector expressing a tumor suppressor, particularly p53, for the treatment of cancer. The Action further contends that Fisher teaches using adenovirus encoding MDA-7 for the

treatment of cancer and administering vectors to tumor cells, which may provide a therapeutic benefit for the treatment of human cancer in general. The Action acknowledges that Fisher does not discuss the details of such therapy. The Action also argues that claims 20-23 and 37-41 have limitations regarding the timing of the combination therapy that neither of the references discusses. It alleges that these limitations, however, fall within the bounds of optimization for a proper therapeutic regimen that a person of ordinary skill in the art would know. It concludes that thus it would have been obvious to one of ordinary skill in the art at the time of the invention to modify the methods taught by Roth by simply substituting p53 with MDA-7 as taught by Fisher. The ordinary skilled artisan is alleged to have been motivated to modify the claimed invention because the combined therapy would maximize the tumor treating effect of any individual therapy alone. Applicants respectfully traverse this rejection.

Three basic criteria must be met to establish a *prima facie* case of obviousness:

(1) "there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings";

(2) "there must be a reasonable expectation of success"; and

(3) "the prior art reference (or references when combined) must teach or suggest all the claim limitations."

MPEP §2142. The present rejection does not meet at least two of these criteria because they do not teach or suggest all of the claim limitations and there was no reasonable expectation of success.

i) Claim limitations not taught by the combination of references

As discussed above, the Fisher patent does not mention angiogenesis. A review of the Roth patent reveals that it too does not mention angiogenesis. The claims recite inhibition of

angiogenesis and consequently, this combination of references does not teach each of the claim limitations.

<p style="text-align:center;">ii)   No reasonable expectation of success</p>

The issue is whether the combination of references provided to the skilled artisan a reasonable expectation of achieving the claimed invention, which is inhibition of angiogenesis by administering a nucleic acid expressing the human MDA-7 polypeptide. As neither reference discusses angiogenesis, the skilled artisan would not have any reason to believe that combining the teachings of the references would provide a way to inhibit angiogenesis in a patient. Accordingly, the skilled artisan had no reasonable expectation of success with respect to the claimed invention. For this reason as well, a proper *prima facie* case is lacking. Applicants respectfully request this rejection be withdrawn.

G.   Provisional Double Patenting Rejection
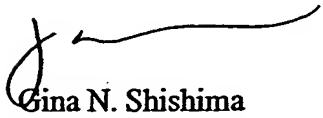
The Action provisionally rejects some of the claims of the application in view of copending U. S. Patent Application No. 09/615,154. Because this rejection is provisional, Applicants will, if necessary, address this rejection once claims in that application or the present application become otherwise allowable.

<p style="text-align:center;">CONCLUSION</p>

Applicants believe that the foregoing remarks fully respond to all outstanding matters for this application. Applicants respectfully request that the rejections of all claims be withdrawn so they may pass to issuance.

Should the Examiner desire to sustain any of the rejections discussed in relation to this Response, the courtesy of a telephonic conference between the Examiner, the Examiner's supervisor, and the undersigned attorney at 512-536-3081 is respectfully requested.

Respectfully submitted,

Gina N. Shishima
Reg. No. 45,104
Attorney for Applicants

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Date: January 30, 2004

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
    Chada et al.

Group Art Unit: 1632

Serial No.: 10/017,472

Examiner: Li, Qian J.

Filed: December 7, 2001

Atty. Dkt. No.: INGN:097US

For: METHODS OF TREATMENT
     INVOLVING HUMAN MDA-7

DECLARATION OF SUNIL CHADA, Ph.D

I, Sunil Chada, declare:

1.   I am the Director of Research and Development at Introgen Therapeutics. I have been working in the field of gene therapy and cancer biology for at least 15 years. My *curriculum vitae* is attached as Exhibit 1.

2.   I am also one of the inventors named on the application identified above, which concerns the melanoma differentiation associated gene (mda-7) and its encoded protein, MDA-7.

3.   The *mda-7* gene was first identified in human melanoma cell lines as a possible tumor suppressor. Jiang *et al.*, *Oncogene* 11:2477-86 (1995). Subsequent studies confirmed that elevated levels of MDA-7 suppressed cancer cell growth *in vitro* and selectively induced apoptosis in human breast cancer cells and inhibited tumorigenicity in nude

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mice. Jiang *et al., Proc. Nat'l. Acad. Sci.* 93:9160-65 (1996); Su *et al., Proc. Nat'l Acad. Sci.* 95:14400-05 (1998).

4.   I understand that the present application contains claims directed to methods of inhibiting angiogenesis involving administering a nucleic acid expressing the human MDA-7 polypeptide, which have been rejected as lacking enablement.

5.   As described in this application, the first 48 amino acids of the full-length sequence may be cleaved to yield a secreted form of the protein. I have done scientific research on the tumor suppressor gene mda-7 and the MDA-7 protein, both the full-length and truncated versions.

6.   In one study concerning the MDA-7 protein, human melanoma cell lines MeWo and WM35 were treated with increasing concentrations of an MDA-7 protein lacking the first 48 amino acids of the full-length sequence. The cell lines were analyzed in triplicate at 12, 24, 48, 72, and 96 hours after treatment using a trypan blue exclusion assay. This truncated MDA-7 protein induced cell killing in melanoma cells (Exhibit 2), but did not induce killing in lung cancer cells.

7.   In another study, different forms of the MDA-7 protein were evaluated in PC3 human prostate cancer cells and H1299 human non-small cell lung carcinoma cells. The different forms (Exhibit 3) included: a full-length MDA-7, an MDA-7 protein lacking its own secretion signal (cytoplasmic version. lacking first 48 amino acids), an MDA-7 targeted to the nucleus (nuclear version), and an MDA-7 lacking its own secretion signal but containing a signal targeting it to the endoplasmic reticulum (ER version). Cells transfected with either the full-length or ER version of MDA-7 showed growth suppression (Exhibit 4). Furthermore, there were higher levels of apoptosis observed in

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cells transfected with the full-length or ER versions, as compared to the cytoplasmic or nuclear versions of MDA-7.

8. Thus, as discussed in paragraph 6, the truncated version of MDA-7 does indeed induce apoptosis as set forth in the specification of this application. Furthermore, as discussed in paragraph 7, a truncated MDA-7 with a heterologous signal sequence suppresses growth and induces apoptosis.

9. Moreover, while the specification provides data regarding an Ad-mda7 construct to express MDA-7 in a eukaryotic cell, another study involved formulating a plasmid with an MDA-7 encoding nucleic acid in a liposome composition. The human mda-7 cDNA was placed under the control of the CMV promoter in a plasmid, which was formulated in a DOTAP:cholesterol complex. Nude mice were injected with human non-small cell lung carcinoma cells (A549 cell line) to produce tumors. Tumors were then treated intratumorally with the DOTAP:Chol-*mda-7* complex (50 µg/dose), resulting in the inhibition of tumor growth as compared to tumors in control animals. Similarly, tumors in nude mice from implantation of fibrosarcoma cells (UV223M cells) (syngeneic tumor model) were also inhibited by intratumoral administration of the DOTAP:Chol-*mda-7* complex. Moreover, when the tumor tissue from these animals were evaluated for CD31, they exhibited reduced levels of staining, which is indicative of reduced vascularization.

10. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such

willful false statements may jeopardize the validity of this application or any patent issued

thereon.

01-29-04

Date

Sunil Chada, Ph.D.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
SUNIL CHADA	DIRECTOR OF RESEARCH AND DEVELOPMENT

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Kings College, University of London London, England	B.Sc. (Honors)	1982	Cell & Molecular Biology
University of California at Los Angeles Los Angeles, CA	M.Sc.	1985	Molecular Biology
University of Massachussetts Medical School Worcester, MA	Ph.D.	1988	Molecular Genetics

A.

B. **Positions and Honors.** List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

PROFESSIONAL EXPERIENCE

1985-1988 Research Associate, Univ. of Massachusetts Medical School, Worcester MA
1988-1991 Research Scientist I, Dept. of Molecular Virology, Viagene Inc., San Diego CA
1991-1993 Research Scientist II, Dept. of Immunobiology, Viagene Inc., San Diego CA
1993-1995 Senior Scientist, Dept. of Immunobiology, Viagene Inc., San Diego CA
1995-1997 Staff Scientist, Chiron Technologies Inc., San Diego CA
1997-pres Director of Research and Development, Introgen Therapeutics, Houston TX
2002-pres Adjunct Faculty, Dept. of Bioimmunotherapy, Division of Cancer Medicine, MD Anderson Cancer Center

Committee Memberships

National Cancer Institute – SBIR/ STTR SRG Reviewer (standing member)
National Cancer Institute – Cancer Chemoprevention (Ad hoc member)
National Cancer Institute – RAID Committee member
Rice University – Advisory Board for NIH and NSF Biotechnology Training Programs
Alliance for Cancer Gene Therapy - Reviewer

C. **Selected peer-reviewed publications (from a total of 68).**

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3) **Chada S**, Whitney C, and Newburger, PE. "Control of expression of the human glutathione peroxidase gene by selenium." In: *OxyRadicals in Molecular Biology and Pathology*, eds. Cerutti P, Fridovich I, and McCord J, UCLA Symposia on Molecular and Cellular Biology Vol. 82. Alan R. Liss, New York, pp.273-288 (1988).

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Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 3a, 3b.

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C. Research Support.

GRANTS AWARDED (from a total of 9)

1. Chada S. "Novel Gene Therapeutic for the Treatment of Lung Cancer" SBIR Grant 1R43CA86587-01 (Funded 03/00). Role: PI. Goals: to evaluate Ad-mda7 as a potential therapeutic for NSCLC

2. Meyn R "Tumor cell radiosensitization by gene drugs" STTR Grant. (Funded 08/00). Role: co-PI. Goal: to evaluate radiosensitization by Ad-p16 and Ad-mda7

3. Grimm EA "Novel gene therapy for Melanoma" STTR grant (Funded 06/01). Role: co-PI. Goals: to evaluate Ad—mda7 as a potential therapeutic for melanoma.

4. Chada S "Combination treatment for breast cancer using Ad-mda7 plus Herceptin". SBIR grant (Funded 07/02). Role: PI. Goals: To evaluate synergy between Ad-mda7 and Herceptin in breast cancer

5. Grimm EA "Phase II clinical trial for Melanoma using INGN 241 (Ad-mda7)" STTR grant (Funded 09/03). Role: co-PI

PATENTS and APPLICATIONS   8 issued patents; 17 applications pending

	Secretion signal	"processed" protein	Myc tag	Predicted Size
(1) FL				25 kD
(2) Cyto				21 kD
(3) Nuc		3X NLS		31 kD
(4) ER	ER signal peptide	ER retention signal		22 kD

Top panel: Y-axis: Colonies (% of control), scale 0 to 120. X-axis: Plasmid Construct — full length, Cyto, nuc, ER.

Bottom panel: Y-axis: % Apoptotic cells, scale 0 to 30. X-axis: Mock, Cytoplasm, Nucleus, Full-Length, ER.

A Single Intramuscular Injection of Recombinant Plasmid DNA Induces Protective Immunity and Prevents Japanese Encephalitis in Mice

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Plasmid vectors containing Japanese encephalitis virus (JEV) premembrane (prM) and envelope (E) genes were constructed that expressed prM and E proteins under the control of a cytomegalovirus immediate-early gene promoter. COS-1 cells transformed with this plasmid vector (JE-4B clone) secreted JEV-specific extracellular particles (EPs) into the culture media. Groups of outbred ICR mice were given one or two doses of recombinant plasmid DNA or two doses of the commercial vaccine JEVAX. All mice that received one or two doses of DNA vaccine maintained JEV-specific antibodies 18 months after initial immunization. JEVAX induced 100% seroconversion in 3-week-old mice; however, none of the 3-day-old mice had enzyme-linked immunosorbent assay titers higher than 1:400. Female mice immunized with this DNA vaccine developed plaque reduction neutralization antibody titers of between 1:20 and 1:160 and provided 45 to 100% passive protection to their progeny following intraperitoneal challenge with 5,000 PFU of virulent JEV strain SA14. Seven-week-old adult mice that had received a single dose of JEV DNA vaccine when 3 days of age were completely protected from a 50,000-PFU JEV intraperitoneal challenge. These results demonstrate that a recombinant plasmid DNA which produced JEV EPs in vitro is an effective vaccine.

Japanese encephalitis (JE) is a mosquito-borne viral disease of major public health importance in Asia. More than 35,000 cases and 10,000 deaths are reported annually (52). *Japanese encephalitis virus* (JEV) is a member of the genus *Flavivirus* in the family *Flaviviridae*. More than 70 species in the *Flavivirus* genus have been genetically and serologically classified (29). Other important human pathogenic flaviviruses include yellow fever, dengue type 1 to 4 (DEN1 to DEN4), tick-borne encephalitis (TBE), and St. Louis encephalitis (SLE) viruses. Vaccination has been an effective mechanism for prevention of flavivirus infection in humans and domestic animals. Three JEV vaccines are in widespread production and use (52). These are inactivated virus from infected mouse brain, inactivated virus from primary hamster kidney cells, and a live attenuated SA14-14-2 vaccine. Only inactivated JEV vaccine, JEVAX, produced in mouse brain is distributed commercially and available internationally (52). Inactivated, mouse brain-derived whole virus vaccine is costly to prepare and carries the risk of allergic reaction to murine encephalitogenic basic proteins or gelatin stabilizer (45; M. M. Andersen, and T. Ronne, Letter, Lancet 337:1044, 1991). Since 1989, an unusual number of systemic reactions characterized by generalized urticaria and/or angioedema following JEVAX immunization have been reported from Australia, Canada, and Denmark (36). A major problem associated with use of the inactivated mouse brain vaccine is the failure to stimulate long-term immunity (39). Multiple immunization is recommended to provide adequate protection (28, 39). The attenuated JEV vaccine, SA14-14-2, is undergoing clinical trials (31). However, because of regulatory issues this vaccine has not found wide acceptance outside the People's Republic of China (11).

Several experimental recombinant virus, attenuated virus, and subunit JEV vaccines have been reported. Recombinant baculovirus vector that contained the JEV envelope (E) protein gene has been used to infect insect cells and produce E protein that has been studied as a biosynthetic immunogen (33). Recombinant vaccinia viruses expressing the JEV genes extending from premembrane (prM) to NS2B proteins have been the most promising candidate vaccines. These candidate vaccines produced extracellular virus-like particles (EPs) in infected cell culture that induced high titers of neutralizing and hemagglutination-inhibiting antibodies and protective immunity in mice (19–21, 47, 54). Recombinant vaccinia viruses expressing the same JEV genes based on the attenuated vaccinia virus strain, NYVAC-JEV, or canarypox, ALVAC-JEV, were tested in phase I human trials (18). In this trial, only 1 in 10 ALVAC-JEV recipients developed detectable viral neutralizing antibody, and vaccinia virus-preimmune recipients had a significantly lower humoral immune response.

Inoculation of animals with purified plasmid vectors (DNA) by the intramuscular (i.m.) or intradermal route leads to expression of the recombinant vector-encoded protein in transfected cells, resulting in stimulation of a protein-specific immune response. Plasmid DNA vaccines provide an alternative to attenuated, inactivated, or virus-vectored subunit vaccines. Flavivirus DNA vaccines for Murray Valley encephalitis, DEN2, JE, SLE, and TBE (Central European encephalitis and Russian spring summer encephalitis) viruses have been developed and tested in the mouse model (4, 17, 24, 30, 38, 49). All of these plasmid DNA constructs contained similar transcriptional regulatory elements and a flavivirus gene cassette. Vaccination of mice with these plasmid DNA vaccines induced a virus-specific antibody response, as detected by enzyme-linked immunosorbent assay (ELISA). However, production of neutralizing antibody leading to 100% protection of vaccinated animals from virus challenge was observed only after multiple immunizations or delivery of DNA to the epidermis by particle

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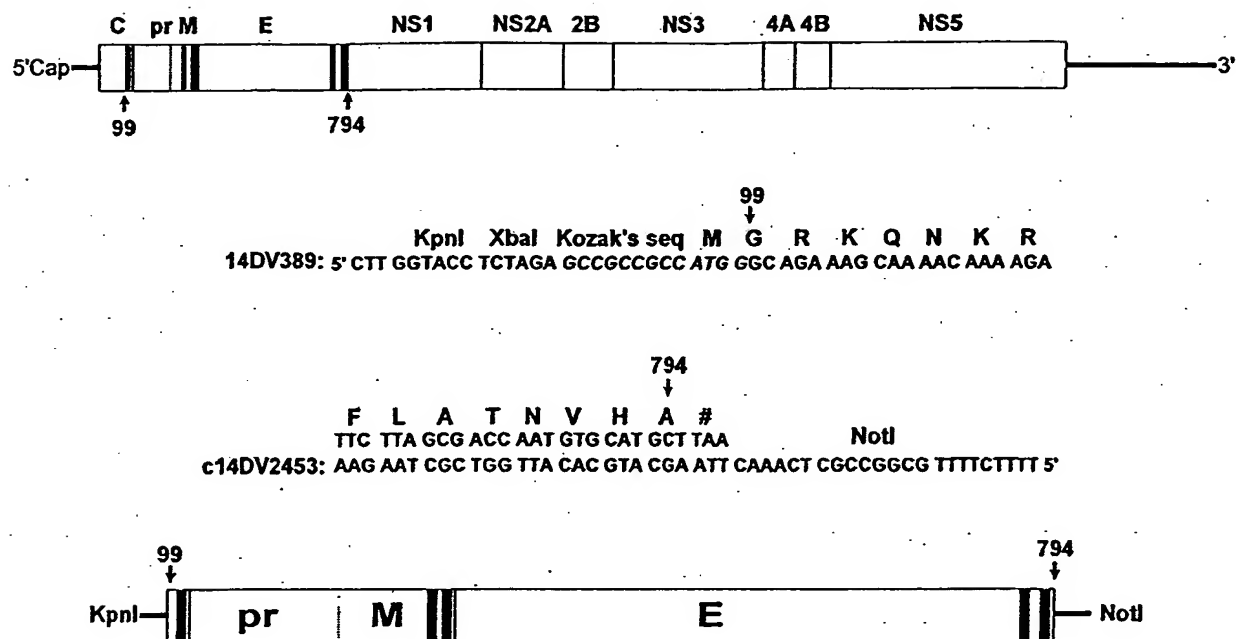
FIG. 1. Map of the JEV genomic structure (top) and the DNA sequence of oligonucleotides used in RT-PCR to construct the transcription unit for the expression of prM-E protein coding regions (bottom). Potential transmembrane helices of viral polyprotein are indicated by blackened areas.

bombardment (4, 24, 49). In this study, we constructed a JEV prM and E gene cassette that incorporates an extended signal peptide sequence at the NH₂ terminus of the prM gene and Kozak's sequence, an optimal translation enhancing element surrounding the AUG site. JEV protein expression was characterized using six different recombinant vectors containing the same insert. The humoral immune response and protection from virulent JEV challenge following immunization with the recombinant plasmid DNAs were compared to findings for the human vaccine, JEVAX, licensed by the U.S. Food and Drug Administration, in outbred ICR mice.

MATERIALS AND METHODS

Cell culture and virus strain. COS-1, COS-7, and SV-T2 cells (1650-CRL, 1651-CRL, and 163.1-CCL; American Type Culture Collection) were grown at 37°C in Dulbecco's modified Eagle medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 7.5% NaHCO₃ (30 ml/liter), penicillin (100 U/ml), streptomycin (100 μg/ml). COS-1 and COS-7 cells were derived from simian virus 40 (SV40) transformed CV1 cells which have an African green monkey kidney cell origin. SV-T2 cells were derived from SV40-transformed mouse fibroblasts. Vero cells were grown under the same conditions except that 5% fetal calf serum without nonessential amino acid was used. C6/36 cells (13) were grown at 28°C in the same medium used for the COS-1 cells. The SA14 strain of JEV, propagated by intracranial inoculation into suckling mouse brain, was used for animal challenges and plaque reduction neutralization tests (PRNT). The SA14 virus used in ELISA and Western blot experiments was propagated in C6/36 cells and purified by ultracentrifugation on 30% glycerol-45% potassium tartrate gradients (37).

Construction of plasmids expressing JEV prM and E gene proteins. Genomic RNA was extracted from 150 μl of SA14 mouse brain JEV by using a QIAamp viral RNA kit (Qiagen, Santa Clarita, Calif.). RNA was adsorbed on a silica membrane, eluted in 80 μl of diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.)-treated water, and used as a template for amplification of JEV prM and E genes. Primer sequences were obtained from the published data (35). A single cDNA fragment containing genomic nucleotides (nt) 389 to 2478 was amplified by reverse transcriptase-mediated PCR (RT-PCR). Restriction enzyme sites for KpnI and XbaI and Kozak's sequence for an optimal translation initiation (25, 26) were engineered at the 5′ terminus of the cDNA by amplimer 14DV389. An in-frame translation termination codon, followed by a NotI restriction site, was introduced at the 3′ terminus of the cDNA by amplimer

c14DV2453 (Fig. 1). A single-tube RT-PCR was performed using a Titan RT-PCR Kit (Roche Molecular Biochemical, Indianapolis, Ind.). The RT-PCR product was purified using a QIAquick PCR purification kit (Qiagen), and the DNA was eluted with 50 μl of 1 mM Tris-HCl (pH 7.5).

All vector constructions and analyses were carried out using standard techniques (46). RT-PCR-amplified cDNA was digested with enzymes KpnI and NotI and inserted into the KpnI-NotI site of eukaryotic expression plasmid vector pCDNA3 (Invitrogen, Carlsbad, Calif.). Electroporation-competent Escherichia coli XL1-Blue cells (Stratagene, La Jolla, Calif.) were transformed by electroporation (Gene Pulser; Bio-Rad Laboratories, Hercules, Calif.) and plated on Luria broth (LB) agar plates that contained carbenicillin (100 μg/ml; Sigma). Clones were picked and inoculated into 3 ml of LB containing carbenicillin (100 μg/ml). Plasmid DNA was extracted from a 14-h LB culture by using a QIAprep Spin Miniprep kit (Qiagen). Automated DNA sequencing was performed as recommended on an ABI Prism 377 DNA sequencer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.). Both strands of the cDNA were sequenced and compared to the published SA14 virus sequence (35).

The pCDNA3 fragment from nt 1289 to nt 3455, which contained the fl-encoded eukaryotic origin of replication (ori), SV40 ori, neomycin coding region, and SV40 poly(A) elements, was deleted by PvuII digestion and then self-ligated to generate plasmid pCBamp. The pCIBamp vector, which contained a chimeric intron insertion at the NcoI-KpnI site of the pCB vector, was constructed by excising the intron sequence from pCI (Promega, Madison, Wis.) by digestion with NcoI and KpnI. The resulting 566-bp fragment was cloned into NcoI-KpnI-digested pCBamp to replace its 289-bp fragment. Figure 2 shows a schematic drawing of plasmids pCDNA3, pCBamp, and pCIBamp.

The DNA fragment containing the JEV coding region in the recombinant plasmid pCDJE2-7, derived from the pCDNA3 vector, was excised by NotI and KpnI or XbaI digestion and cloned into the KpnI-NotI sites of pCB, pCIB, pCEP4 (Invitrogen), and pREP4 (Invitrogen) and into the SpeI-NotI site of the pRc/RSV (Invitrogen) expression vector to create pCBJE1-14, pCIBJES14, pCEJE, pREJE, and pRCJE, respectively. Both strands of the cDNA from each plasmid vector were sequenced, and recombinant clones with a correct nucleotide sequence were identified. Plasmid DNA for in vitro transformation or mouse immunization was purified by anion-exchange chromatography using an Endo-Free Plasmid Maxi kit (Qiagen).

IFA. Expression of JEV-specific gene products by the various recombinant expression plasmids was evaluated by indirect immunofluorescence antibody assay (IFA) in the transient expression system using COS-1, COS-7, and SV-T2 cells. For transformation, cells were grown to 75% confluence in 150-cm² culture flasks, trypsinized, and resuspended in 4°C phosphate-buffered saline (PBS) to a final density of 1 × 10⁷ to 2 × 10⁷ cells/ml. Five hundred microliters of cell suspension was then electroporated with 10 μg of plasmid DNA, using a Bio-Rad Gene Pulser II set at 250 V and 960 μF. Cells were diluted with 25 ml of fresh medium after electroporation and seeded into one 75-cm² flask. Forty-eight hours after transformation, the medium was removed, and the cells were

TATA box   KpnI   BamHI   EcoRI   EcoRV   NotI

TATATAA...(92nt)...GGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCTGCAGATATCCATCACACTGGCGGCCGC...BHG-poly(A)
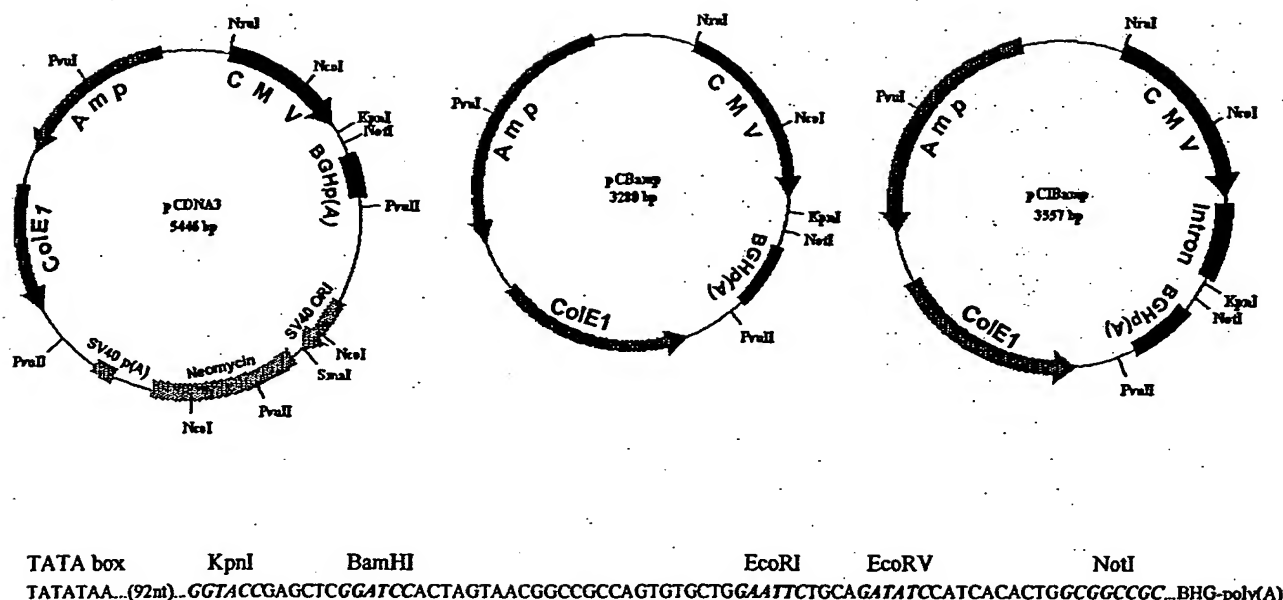
FIG. 2. Schematic representations of plasmid vectors pCDNA3, pCBamp, and pCIBamp. These plasmids include the CMV promoter/enhancer element, BGH poly(A) signal and transcription termination sequence [BHGp(A)], ampicillin resistance gene (Amp), and ColE1 ori for selection and maintenance in *E. coli*. The fI ori for single-stranded rescue in *E. coli* cells, SV40 ori, neomycin coding region, and SV40 poly(A) [SV40 p(A)] sequences were deleted from pCDNA3 to generate pCBamp. An intron sequence was inserted in the *NcoI-KpnI* site of pCBamp to generate pCIBamp. The multiple cloning site for the insertion of JEV genes, located between the TATA box of the CMV promoter/enhancer and BHG poly(A) site, is shown.

trysinized and resuspended in 5 ml of PBS with 3% normal goat serum. Ten-microliter aliquots of the cell suspension were then spotted onto slides, air dried, and fixed with acetone at 4°C for 10 min. Immunofluorescent mapping of the E protein-specific epitopes was performed using a panel of murine monoclonal antibodies (MAbs) (15, 42, 55) and JEV-specific hyperimmune mouse ascitic fluid (HIAF). All antibodies were tested at 1:400 dilution in PBS.

Selection of an in vitro-transformed stable cell line constitutively expressing JEV-specific gene products. COS-1 cells transformed with 10 µg of pCDJE2-7 DNA by electroporation were incubated in nonselective culture medium for 24 h and then treated with neomycin (G418; 0.5 mg/ml; Sigma). G418-resistant colonies, which became visible after 2 to 3 weeks, were cloned by limited dilution in G418-containing medium. Expression of the JEV proteins was determined by IFA using JEV HIAF. One IFA-positive (JE-4B) and one IFA-negative (JE-5A) clone were selected for further analysis and maintained in medium containing 200 µg of G418 per ml. These stably transformed cells secreted antigen in the form of EPs (A. Hunt and G. J. Chang, unpublished data).

Antigen capture ELISA for detection of E protein secreted into culture fluid. The antigen capture ELISA, a modification of the procedure described by Guira-khoo et al. (8), was used to detect E protein from transiently transformed cells or JE-4B culture fluid. Flavivirus group-reactive MAb 4G2 was used to capture the JEV antigens (7). The 4G2-captured antigen was detected using horseradish peroxidase-conjugated MAb 6B6C-1 by incubation for 1 h at 37°C. Enzyme activity on the solid phase was detected with 3,3',5,5'-tetramethylbenzidine ELISA substrate (Life Technologies, Grand Island, N.Y.); the reaction was stopped with the addition of 2 M H₂SO₄, and the optical density was measured at 450 nm.

Mouse experiments. Three-day-old mixed-sex or 3-week-old female ICR outbred mice were vaccinated i.m. with 50 or 100 µg of plasmid DNA at a concentration of 1 µg/µl in PBS or subcutaneously (s.c.) with 1/10 or 1/5 of the adult human dose of JEVAX (manufactured by the Research Foundation for Microbial Disease of Osaka University and distributed by Connaught Laboratories, Swiftwater, Pa.). The chloramphenicol acetyltransferase (CAT) protein expression plasmid pCDNA3/CAT (Invitrogen) was used as the vaccination control. Selected groups of mice were boosted 3 weeks later with an additional dose of plasmid vaccine or JEVAX. Mice were bled from the retro-orbital sinus; serum samples were evaluated for JEV antibody by ELISA and Western blotting using purified JEV and by PRNT.

Mice vaccinated at 3 days of age were challenged intraperitoneally (i.p.) 7 weeks postvaccination with JEV strain SA14 (50,000 PFU/100 µl) and observed for 3 weeks. To evaluate passive protection by maternal antibody, pups were obtained from mating of nonimmunized males with immunized females 9 weeks following their vaccination with plasmid DNA at 3 weeks of age. Pups were challenged by the i.p. route 3 to 15 days after birth with SA14 virus (5,000 PFU/100 µl) and observed daily for 3 weeks. Postchallenge serum was collected from survivors and tested for reactivity with JEV antigens by ELISA and Western blotting.

Serological tests. Postvaccination and postchallenge serum samples were tested for the ability to bind to purified JEV by ELISA, neutralize JEV infectivity by PRNT, or recognize JEV proteins by Western blotting (12, 41, 48). The PRNT assay was performed by incubating ~200 PFU of SA14 virus in 100 µl of Dulbecco's modified Eagle medium containing 5% bovine serum albumin and 20 mM HEPES buffer (pH 8.0) with serial twofold dilutions of serum specimens, started at 1:10, in 100 µl of the same buffer in 96-well trays at 4°C overnight. Serum specimens were heat inactivated at 56°C for 30 min before use. Duplicate 100-µl aliquots were assayed for infective virus by plaque formation on Vero cell monolayers. The percent plaque reduction was calculated relative to virus controls without serum. Titers were expressed as the reciprocal of serum dilutions yielding a 90% reduction in plaque number (PRNT₉₀).

RESULTS

Effect of the promoter and poly(A) signal on the efficiency of JEV prM and E protein expression. Four eukaryotic cell expression plasmids that contained the JEV coding region extending from genomic nt 390 to nt 2478 were constructed. This region of the genome encoded the prM and E genes. The Kozak sequence for the eukaryotic translation initiation site (underlined) of −9 to +4, GCCGCCGCCATGG, at the 5' terminus (2, 25, 26, 27) and the in-frame translation terminat ion sequence at the 3' terminus of cDNA were incorporated directly into cDNA by RT-PCR using viral RNA as a template. Transcription of the JEV genes in plasmid pCDJE2-7 was controlled by the human cytomegalovirus (CMV) early IA gene promoter/enhancer. The resulting mRNA is terminated and stabilized by a bovine growth hormone (BGH) transcript ion terminator and a poly(A) signal, respectively. The transcriptional control elements in pREJE were replaced by the Rous sarcoma virus (RSV) long terminal repeat promoter and SV40 poly(A). The pCEJE and pRCJE plasmids contain CMV plus SV40 poly(A) and RSV plus BGH poly(A), respectively (Table 1).

To determine the influence of the promoter and poly(A) elements on JEV prM and E protein expression, recombinant plasmids pCDJE2-7, pCEJE, pRCJE, and pREJE were ini-

TABLE 1. Transient expression of JEV prM and E proteins by various recombinant plasmids in two transformed cell lines

Name	Promoter	Intron	Poly(A)	Ori	Recombinant plasmid	IFA intensity/% positive[a] COS-1	IFA intensity/% positive[a] COS-7
pCDNA3	CMV	No	BGH	SV40	pCDJE2-7	3+/40	3+/35
pCBamp	CMV	No	BGH	No	pCBJE1-14	3+/45	ND
pCIBamp	CMV	Yes	BGH	No	pCIBJES14	3+/39	ND
pCEP4	CMV	No	SV40	OriP	pCEJE	2+/4	2+/3
pREP4	RSV	No	SV40	OriP	pREJE	1+/3	1+/2
pRc/RSV	RSV	No	BGH	SV40	pRCJE	1+/3	1+/3
pCDNA3	CMV	No	BGH	SV40	pCDNA3/CAT	−	−

[a] Various cell lines were transformed with pCDNA3/CAT (negative control), pCDJE2-7, pCBJE1-14, pCIBJES14, pCEJE, pREJE, or pRCJE. Cells were trypsinized 48 h later and tested by IFA with JEV HIAF. Data are presented as the intensity (scale of 1+ to 4+) and percentage of IFA-positive cells. pCDNA3/CAT-transformed cells were used as the negative control. ND, not determined. −, negative.

tially tested for the ability to express JEV prM and E proteins following transformation of various mammalian cells. COS-1, COS-7, and SV-T2 cells were transiently transformed with equal amounts of pCDJE2-7, pCEJE, pRCJE, or pREJE plasmid DNA. The SV-T2 cell line was excluded from further testing after preliminary results showed that less than 1% of pCDJE2-7-transformed SV-T2 cells were expressing JEV antigen.

JEV antigens were expressed in COS-1 and COS-7 cells transformed by all four recombinant plasmids, thus confirming that the CMV or RSV promoter and BGH or SV40 poly(A) elements were functionally active. However, the percentage of transformed cells and the level of JEV antigens expressed, as determined by the number of IFA-positive cells and IFA intensity, respectively, differed significantly (Table 1). A significantly higher percentage of pCDJE2-7-transformed COS-1 cells expressed JEV proteins with greater IFA intensity at a level equal to that observed with JEV-infected cells. Cells transformed with the pCEJE, pREJE, or pRCJE vector, on the other hand, showed a lower percentage of antigen-expressing cells as well as a lower IFA intensity. Vectors containing the CMV promoter and BGH poly(A) were selected for further analysis (Fig. 2).

To determine whether the enhanced expression of JEV proteins by the pCDJE2-7 vector was influenced by the SV40 ori, we constructed the pCBJE1-14 vector in which a 2,166-bp fragment containing the f1 ori, SV40 ori, neomycin coding region, and SV40 poly(A) elements was deleted. A chimeric intron was then inserted into pCBJE1-14 to generate pCIBJES14. Plasmid pCIBJES14 was used to determine whether the expression of JEV proteins could be enhanced by an intron sequence. Following transformation, both pCBJE1-14 and pCIBJES14 vectors resulted in cells expressing levels of JEV proteins similar to that observed with the pCDJE2-7 vector (Table 1). These results indicated that expression of the JEV proteins was influenced only by the transcriptional regulatory elements encoded in the recombinant plasmid. Neither the SV40 ori nor the intron sequence enhanced JEV protein expression in the cells used.

Epitope mapping of E protein expressed by a stably transformed cell line constitutively expressing JEV-specific gene products. Authenticity of the JEV E protein expressed by the JE-4B clone was demonstrated by epitope mapping by IFA using a panel of JEV E-specific murine MAbs. JEV HIAF and one irrelevant mouse ascitic fluid were used as positive and negative antibody controls, respectively. Four JEV-specific, six flavivirus subgroup-specific, and two flavivirus group-reactive MAbs reacted similarly with the 4B clone and with JEV-infected COS-1 cells (Table 2).

Detection of JEV E protein secreted by the JE-4B COS-1 cell line. An antigen capture ELISA, employing flavivirus group-reactive, anti-E MAbs 4G2 and 6B6C-1, was used to detect JEV E proteins that were secreted into the culture fluid by the COS-1 cell clone JE-4B. Antigen could be detected in the culture fluid the first day following seeding of the cells with maximum ELISA titers that ranged from 1:16 to 1:32.

Comparison of immune responses in mice vaccinated with pCDJE2-7 genetic vaccine and JEVAX. Plasmid pCDJE2-7 was used as a nucleic acid vaccine to induce an antibody response in mice by immunizing groups of five 3-week-old female ICR outbred mice. Mice were bled at 3, 6, 9, 23, 40, and 60 weeks after immunization, and antibody titers were determined by ELISA or by PRNT. As expected, sera from animals in the pCDNA3/CAT control group did not contain JEV antibody. All animals immunized with pCDJE2-7 and JEVAX seroconverted by 3 weeks after the first vaccination (Table 3). The antibody titers were similar irrespective of the number of doses

TABLE 2. Epitope mapping of E protein expressed by JE-4B, a pCDJE2-7 stably transformed clone of COS-1 cells, with JEV-reactive antibodies[a]

MAb or antiserum	Biological activity of MAb Specificity	Biological activity of MAb Biological function	IFA intensity of cells JEV infected	IFA intensity of cells 4B
MAbs				
MC3	JEV specific		2+	2+
2F2	JEV specific	HI, N	4+	4+
112	JEV specific		4+	4+
503	JEV specific	N	4+	3+
109	Subgroup	HI	2+	1+
N.04	Subgroup	HI, N	3+	4+
201	Subgroup		1+	1+
203	Subgroup		4+	3+
204	Subgroup		2+	2+
301	Subgroup	HI	2+	2+
504	Flavivirus		4+	4+
6B6C-1	Flavivirus		2+	2+
3B4C-4	VEE		−	−
HIAF				
Anti-JEV			4+	3+
Anti-WEE			−	−
PBS			−	−

[a] VEE, Venezuelan equine encephalomyelitis virus; WEE, Western equine encephalomyelitis virus. −, negative.

TABLE 3. Persistence of the immune response in mice (five per group) immunized with pCDJE2-7 or JEVAX

Inoculation[a]	ELISA titer (log$_{10}$)						PRNT$_{90}$ titer		
	3[b]	6	9	23	40	60[c]	3	6	9
pCDJE2-7									
1 dose	2.6–3.2	3.8–5.0	3.8–4.4	>3.2	>3.2	2.4, 2.4, 3.8, 4.4	<20	20	40–160
2 doses	2.6–3.8	4.4	3.8–4.4	>3.2	>3.2	2.6, 3.8, 3.8	<20	20–40	40–160
JEVAX, 2 doses	2.6–3.8	4.4–5.0	3.8–5.6	>3.2	>3.2	<2, <2, <2, 4.4	<20	20–40	20–160
pCDNA3/CAT, 2 doses	<100	<100	<100	ND[d]	ND	ND	<20	<20	<20

[a] Three-week-old mice were inoculated i.m. with one or two 100-μg doses of plasmid DNA or twice s.c. with one-fifth of the human dose of JEVAX.
[b] Weeks postimmunization.
[c] Individual serum titers.
[d] ND, not determined.

of pCDJE2-7 or JEVAX given. Mouse serum samples collected 9 weeks after immunization were also tested by Western blotting using purified JEV. Serum specimens from DNA-vaccinated mice, which had reactivity similar to that of JEV HIAF, detected E and prM proteins (Fig. 3). However, mouse serum from JEVAX-immunized mice reacted only with E protein. Comparable ELISA antibody titers were maintained in DNA-vaccinated groups for up to 60 weeks, at which time the experiment was terminated. Only one of four mice in the JEVAX group remained JEV antibody positive at 60 weeks postinoculation. These results demonstrated that one dose of JEV-specific nucleic acid vaccine was more effective in maintaining JEV antibody levels in mice than the commercially available vaccine JEVAX.

Comparison of various nucleic acid vaccine constructs and JEVAX for ability to induce JEV-reactive antibody in different age groups of mice. Similar amounts of JEV protein were expressed by COS-1 cells transformed by either pCDJE2-7, pCBJE1-14, or pCIBJES14. JEV antibody induction by these nucleic acid constructs was compared to results for JEVAX in two different age groups of mice. Three-day-old mixed-sex or 3-week-old female ICR outbred mice, 10 per group, were vac-

cinated i.m. with 50 or 100 μg of plasmid DNA or s.c. with 1/10 or 1/5 of the adult human dose of JEVAX, respectively. Serum specimens were collected at 7 weeks after immunization and tested at 1:400 or 1:1,600 by ELISA. Ninety to 100% of all 3-week-old mice that received pCBJE1-14, pCDJE2-7, pCIBJES14, or JEVAX had antibody titers of ≥1:1,600. However, a significant difference in antibody response was observed in 3-day-old groups that received various vaccines. None of the 3-day-old JEVAX-vaccinated mice had antibody titers higher than 1:400. All 3-day-old mice vaccinated with pCBJE1-14 had antibody titers higher than 1:1,600. Seroconversion of 100% was observed at 1:400 in 3-day-old mice that received pCDJE2-7 or pCIBJES14, but only 60% of both mouse groups were positive at 1:1,600. pCBJE1-14 was the most effective of three DNA constructs tested. The minimum dose of this DNA construct capable of providing 100% seroconversion (1:400 by ELISA) by i.m. immunization in 3-week-old mice was determined to be 25 μg (data not shown).

Protective immunity conferred by the nucleic acid vaccine. Mice immunized at 3 days of age were challenged by the i.p. route at 7 weeks postvaccination with the SA14 strain of JEV (50,000 PFU/100 μl) and observed for 3 weeks. One hundred percent of the animals that received various nucleic acid vaccine constructs were protected. In contrast, only 40 and 30% of mice that received JEVAX and pCDNA3/CAT, respectively, survived virus challenge (Fig. 4). These results suggested that the DNA vaccine could be effective as a neonatal vaccine. In contrast, JEVAX was not as effective in neonatal animals.

FIG. 3. JEV-specific reactivity of prechallenge and postchallenge serum samples obtained from mice immunized with DNA vaccine or JEVAX. Serum specimens collected from the mice used in the experiments represented in Tables 3 and 4 were randomly selected and tested at 1:1,000 dilution by Western blot analysis using purified JEV as the antigen. pCDJE2-7x2-S was the serum from one of the mice challenged at 4 days of age (Table 4). NMAF, 4G2-AF, and JEV HIAF were the mouse ascitic fluids included as normal mouse, E-specific, and JEV hyperimmune controls, respectively.

FIG. 4. Postchallenge survival rates of mice (10 per group) that were immunized with pCDJE2-7, pCBJE1-14, pCIBJES14, pcDNA3/CAT, or JEVAX at 3 days of age and challenged i.p. with 50,000 PFU of JEV (SA14) 7 weeks postimmunization. A P value of 0.003 was obtained by Fisher's exact test when the survival rate of the JEV DNA-immunized groups was compared with that of the pcDNA3/CAT or JEVAX group.

TABLE 4. Ability of maternal antibody from JEV nucleic acid-vaccinated female mice to protect their pups from fatal JE

Vaccinated mother[a]		JEV-challenged pups			
Vaccine	PRNT$_{50}$	Age (days)	No. of survivors/ total in litter	Avg survival time (days)	ELISA[b]
1 × pCDJE2-7	40	4	0/11	5.27	
2 × pCDJE2-7	80	4	12/12	NA[c]	12/12
2 × JEVAX	20	3	0/16	4.75	
2 × pCDNA3/CAT	<10	5	0/14	4.00	
1 × pCDJE2-7	20	15	5/11	10.0	5/5
2 × pCDJE2-7	40	14	8/12	13.75	7/8
2 × JEVAX	80	13	5/5	NA	5/5
2 × pCDNA3/CAT	<10	14	0/14	6.14	

[a] Mice were inoculated i.m. with one or two 100-μg doses of pCDJE2-7 DNA or twice s.c. with one-fifth of the adult human dose of JEVAX. Serum samples were collected 9 weeks postvaccination for PRNT testing prior to mating with nonimmune male.

[b] Number of JEV ELISA antibody-positive animals (titer ≥ 1:400)/number of survivors. Serum specimens were collected for testing 12 weeks after challenge.

[c] NA, not applicable.

Passive protection of neonatal mice correlated with the maternal antibody titer. Female 3-week-old ICR mice were vaccinated with one or two doses of pCDJE2-7 plasmid DNA (100 μg/100 μl) or twice with one-fifth of the adult human dose of JEVAX. For evaluation of passive protection by maternal antibody, pups were obtained from matings of experimental females with nonimmunized male mice. Pups were challenged by the i.p. route at 3 to 5 or 13 to 15 days after birth with SA14 virus (5,000 PFU/100 μl). Survival rates and average survival time correlated with the maternal neutralizing antibody titers (Table 4). One hundred percent of pups nursed by mothers with a PRNT of 1:80 survived viral infection regardless of the type of vaccine received by the mothers. None of the pups from mothers which received pCDNA3/CAT plasmid DNA survived (Table 4). Partial protection (45% [5 of 11 pups] to 67% [8 of 12 pups]) was observed in older pups that were nursed by the mothers which had serum PRNT titers of 1:20 and 1:40, respectively. However, none of the 3-day-old pups survived virus challenge when the mothers had a serum PRNT titer of 1:20 or 1:40. Maternally transferred antibody can only be detected in the circulation of the young mouse up to 40 days after birth. An appreciable level of maternally derived antibody is maintained in the circulation of the young mouse 24 days or more postpartum (1). JEV ELISA antibody detected in the serum of 97% (29 of 30) of the postchallenge pups at 12 weeks after virus challenge was unlikely to be residual maternally transferred antibody. The presence of JEV antibody in the surviving pups challenged at 3 to 4 or 13 to 15 days of age strongly suggested that maternal antibody did not provide sterilizing immunity to the pups. It also indicated that 3- to 4- or 13- to 15-day-old m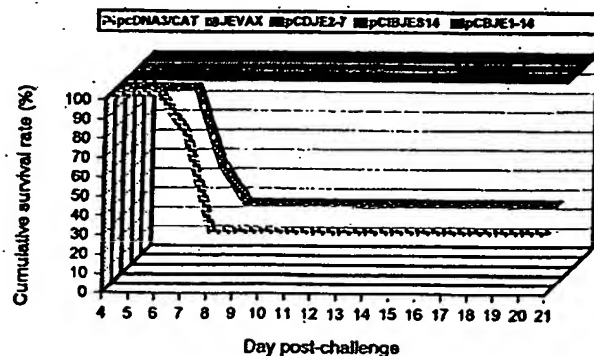ice could mount an immune reaction to a live-virus challenge. Partial protection in older pups could be explained by the opportunity to accumulate a large quantity of passive antibody due to the length of nursing time before challenge. One randomly selected postchallenge serum sample also reacted with prM and E proteins by Western blotting (Fig. 3).

DISCUSSION

The flavivirus virion contains a capsid protein (C), a membrane protein (M), and an E protein. The prM MAbs, exhibiting weak or undetectable neutralizing activity in vitro, can provide passive protection following DEN2 virus challenge (16). However, the E protein plays a dominant role in generating neutralizing antibodies and providing protective immunity in the host. Passive transfer of JEV E-specific neutralizing MAbs has been shown to protect recipients from JEV-induced fatal encephalitis (3, 16, 32, 55). Antigenic and structural analysis using various panels of MAbs has shown that most of the E protein epitopes that elicit virus-neutralizing antibodies are conformationally dependent (9, 40). Coexpression of both proteins as type I transmembrane proteins is essential to maintain proper E conformation and prevent the E protein from undergoing irreversible, low-pH-catalyzed conformational changes (8–10, 19, 50). A 2-kb genomic region, from the internal signal peptide at the carboxyl terminus of C to the transmembrane domain at the carboxyl terminus of the E gene, is essential for expressing authentic proteins. These authentic prM and E proteins are able to self-assemble into virus-like particles in cells infected by either recombinant vaccinia virus or alphavirus vector or in cells transformed by recombinant plasmid DNA (4, 19, 22, 48; Hunt and Chang, unpublished data).

A gene cassette including the elements listed above was amplified from SA14 virus by RT-PCR in the present study. Optimal sequence composition surrounding the translation initiation site (−9 to +4) was incorporated into the 14DV398 amplifying primer (2, 26, 27) (Fig. 1). Recombinant plasmids containing the CMV early gene promoter/enhancer and the BHG poly(A) terminator as transcription regulatory elements expressed JEV proteins with the highest efficiency in three different cell lines. Protein expression and the serological response of mice immunized with DNA vaccine were not influenced by the presence or absence of the SV40 ori or an intron sequence in recombinant plasmids. Virus-specific proteins, secreted into culture medium, could be detected by antigen capture ELISA as early as 48 h after plasmid transformation (data not shown). The authenticity of the E protein produced by the pCDJE2-7 stably transformed cell line, JE-4B, was demonstrated by MAb epitope mapping.

Vaccine potential and characteristics of various eukaryotic plasmids that express flavivirus prM and E proteins are summarized in Tables 5 and 6. All constructs listed had the same transcriptional control elements and similar viral gene cassettes. DEN2 plasmid, which contains prM and 91% of E, is the only exception (Table 6). The JEV DNA vaccine reported in this study is the only construct that stimulated complete protective immunity in mice by a single dose of vaccine given by the i.m. route (Table 5). Sequences surrounding the translation initiation site and the composition of the signal peptide preceding the prM protein are the two major differences among the constructs that may contribute to increasing the vaccine potential of our construct (Table 6). Conserved features of the sequences which flank vertebrate translation initiation sites include a strong preference for purine at the −3 position; a higher frequency of G at positions −9, −6, −3, and +4; and a preference for A or C at positions −5, −4, −2, and −1 (2). Instead of the sequence used in previous publications, the sequence used in our construct was −9 · GCCGCCGCC ATGG, which fits the general criteria listed above. Although less than 1% of eukaryotic mRNA sequences exhibit this sequence, the experimental data have suggested that this sequence provides exceptionally high levels of translation potential (2, 26).

Signal peptides determine translocation and orientation of inserted protein, hence the topology of prM and E. Signal peptide differences in our plasmid construct may account for the efficient translocation and correct topology, thus increasing prM and E secretion. A machine-learning program using neu-

TABLE 5. Vaccine potential of various eukaryotic plasmids that express flavivirus prM and E proteins[a]

Virus	In vitro secretion of EPs	Immunization			Protection from virus challenge	Reference
		Dosage	Route/method	Neutralizing antibody[b]		
JE	Yes	25–100 μg × 1	i.m./needle	Yes $(1:20–1:160_{90\%})$	100%	This report
	ND	100 μg × 2	i.m./needle	No	Partial	30
	ND	10–100 μg × 2	i.m. or i.d./needle	Yes $(1:10–1:20_{90\%})$	100%	24
MVE	Yes	100 μg × 4	i.m./needle	ND	Partial	4
	Yes	1–2 μg × 2–4	i.d./gene gun	Yes $(80–320_{50\%})$	100%	4
SLE	ND	100 μg × 2	i.m./needle	No	Partial	38
CEE	ND	1 μg × 1–2	i.d./gene gun	Yes $(1:100–1:1,600_{80\%})$	100%	49
RSSE	ND	1 μg × 1–2	i.d./gene gun	ND	100%	49
DEN2	ND	200 μg × 3	i.d./needle	Yes $(1:10–1:320_{50\%})$	None	17

[a] MVE, Murray Valley encephalitis; CEE, Central European encephalitis; RSSE, Russian spring-summer encephalitis; i.d., intradermal; ND, not done.
[b] Plaque reduction neutralization titer followed by percentage reduction endpoint used in the test.

ral networks trained on eukaryotes (SignalP-NN at http://www.cbs.dtu.dk/services/) was applied to test the efficiency of the prM signal peptide sequence in the different plasmid constructs (34) (Table 6). The most probable location and orientation of transmembrane helices in the prM-E protein were then determined by a hidden Markov model-trained computer program (6 [TMHMM at http://www.cbs.dtu.dk/services/]). SignalP-NN searches correctly predicted the signal peptidase cleavage site of all constructs. However, a considerable difference in cleavage potential (C score, between 0.578 and 1.000) was observed (Table 6). Cleavage potential differences may be influenced by the amino acid composition and length of the h region in various constructs (44).

The TMHMM program correctly predicted five transmembrane helices encoded in the prM-E protein. Significant difference in the probable orientation of the first transmembrane helix was observed in three JEV constructs (Fig. 5). In our pCDJE2-7 construct, the first 12 amino acids of the n region form a short loop in the cytoplasmic side that causes the following h region (transmembrane helix) to be inserted in a tail orientation. Secretion of JEV protein could be detected by antigen capture ELISA in pCDJE2-7 transient expression studies in which less than 5% of the cells were positive by IFA (data not shown). Thus, there is a high probability that prM and E proteins expressed by pCDJE2-7 would be expressed in the correct orientation, as type I transmembrane proteins (Fig. 5A). There is also a high probability that the prM protein of pcDNA3JEME could be expressed as a type II membrane protein with its transmembrane h region inserted in a head orientation because of the absence of positively charged amino acids in its n region (Fig. 5B). Efficient protein synthesis in

conjunction with correct topology of expressed prM and E (Fig. 5A) would most likely enhance EP formation and secretion in transformed cells.

Another characteristic that could explain the excellent vaccine potential of our JEV construct is its ability to produce EPs which have a virus-like polymeric structure that enhances antigenic stability and provides a high-density presentation to antigen-presenting cells, such as macrophages, dendritic cells, and Langerhans cells (5). When DNA is given by the i.m. route, the majority of antigen is expressed by non-antigen-presenting muscle cells. The efficacy of a DNA vaccine is therefore dependent on transfection of antigen-presenting cells or to reprocessing of antigen derived from other cells. Muscle cells transfected by our construct could conceivably synthesize and secrete EPs, which are highly immunogenic and have been shown to elicit good cellular and humoral responses (22, 23).

Genetic JEV vaccine that induced a completely protective immunity in neonatal mice and a maternally transferable protective immunity in young adult mice by a single i.m. immunization was demonstrated in this study. Additional studies are planned to address the effectiveness of a DNA vaccine in overcoming the potential influence of maternally transferred flavivirus antibodies on the induction of JEV antibody in neonatal mice.

Immunization of pigs is a theoretical means of interrupting transmission and amplification of JEV and thereby preventing human infections (43). The JEV DNA vaccine could also be used as a veterinary vaccine in pregnant sows to prevent JEV-induced stillbirth and abortion (51, 53). Maternally transferred antibody could also interrupt piglets as the JEV-amplifying host and thus reduce human infection.

TABLE 6. Characteristics of various eukaryotic plasmids expressing flavivirus prM and E proteins

Virus[a]	Plasmid	Sequence surrounding translation initiation site	Amino acids preceding prM protein[b]	SP potential (C score)[c]	Reference
JE	pCDJE2-7	−9•GCCGCCGCCATGG•+4	MGRKQNKRGGNEGSIMWLASLAVVIACAGA /MKL	Yes (0.921)	This report
	pJME	−9•GGCTCAATCATGG•+4	MWLASLAVVIACAGA /MKL	Yes (0.578)	30
	pCDNA3JEME	−9•GAATTCACCATGG•+4	MNEGSIMWLASLAVVIACAGA /MKL	Yes (0.921)	24
MVE	pCDNA3.prM-E	−9•TGATTTCAAATGT•+4	MSKKRGGSETSVLMVIFMLIGFAAA /LKL	Yes (0.819)	4
SLE	pSLE1	?	?LDTINRRPSKKRGGTRSLLGLAALIGLASS /LQL	Yes (0.709)	38
DEN2	p1012D2ME	?	?AGMIIMLIPTVMA /FHL	Yes (0.646)	17
TBE	SV-PE$_{eq}$	−9•GCGGCCGCCATGG•+4	MVGLQKRGKRRSATDWMSWLLVITLLGMTLA /ATV	Yes (1.000)	48
RSSE	pWRG7077	−9•GTAGACAGGATGG•+4	MGWLLVVVLLGVTLA /ATV	Yes (0.762)	50
CEE	pWRG7077	−9•ACGGACAGGATGG•+4	MSWLLVITLLGMTIA /ATV	Yes (0.609)	50

[a] Abbreviations are as given in Table 5, footnote a.
[b] Single amino acid code. Positively charged amino acid is indicated by bold letter. Signal peptidase cleavage site is indicated by /.
[c] Cleavage potential of signal peptide (SP) predicted by SignalP-NN at http://www.cbs.dtu.dk/services (34).
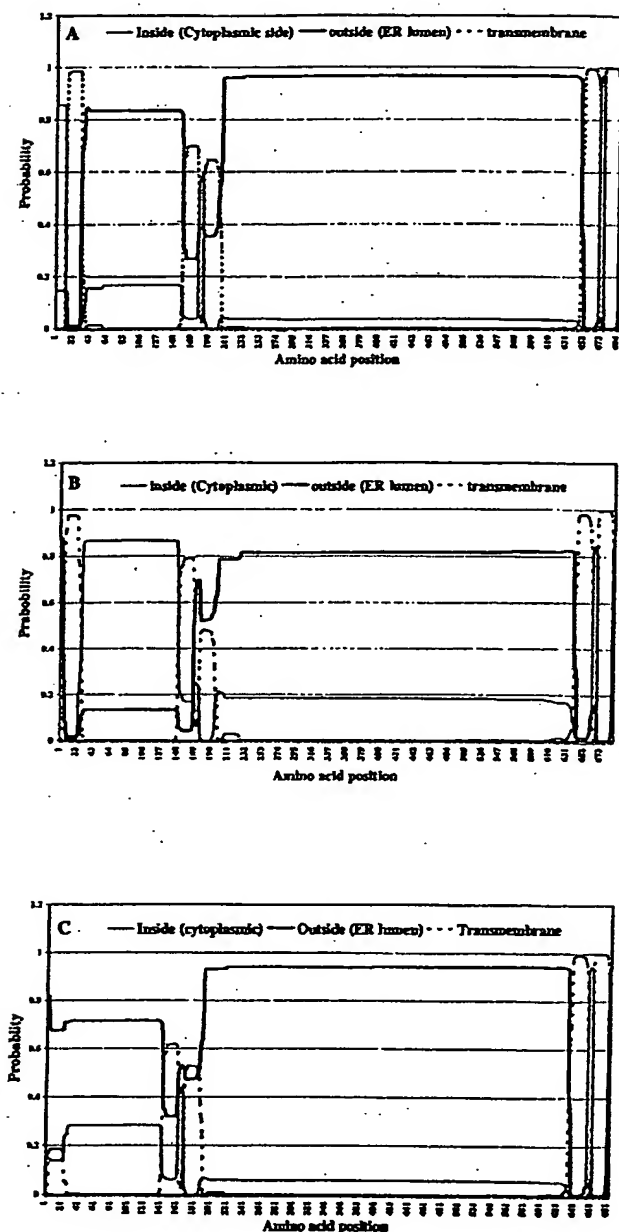
FIG. 5. Graphic representation, generated by the TMHMM program, indicating probable orientations of five transmembrane helices in the prM-E protein expressed by pCDJE2-7 (A), pcDNA3JEME (B), and pJME (C). ER, endoplasmic reticulum.

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Sex Differences in Seoul Virus Infection Are Not Related to Adult Sex Steroid Concentrations in Norway Rats

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Field studies of hantavirus infection in rodents report that a higher percentage of infected individuals are males than females. To determine whether males were more susceptible to hantavirus infection than females, adult male and female Long Evans rats (*Rattus norvegicus*) were inoculated with doses of Seoul virus ranging from 10^{-4} to 10^{6} PFU. The 50% infective doses (ID_{50}) were not significantly different for male and female rats ($10^{0.05}$ and $10^{0.8}$ PFU, respectively). To determine whether sex differences in response to infection were related to circulating sex steroid hormones, sex steroid concentrations were manipulated and antibody responses and virus shedding were assessed following inoculation with the ID_{90}. Regardless of hormone treatment, males had higher anti-Seoul virus immunoglobulin G (IgG) and IgG2a (i.e., Th1) responses than females and IgG1 (i.e., Th2) responses similar to those of females. Males also shed virus in saliva and feces longer than females. Manipulation of sex steroids in adulthood did not alter immune responses or virus shedding, suggesting that sex steroids may organize adult responses to hantavirus earlier during ontogeny.

Hantaviruses are negative-sense RNA viruses (family *Bunyaviridae*) encompassing over 20 different viruses that are each carried by a different host species, with rodents serving as the primary reservoirs (18). Field surveys of several rodent species, including brush mice, deer mice, harvest mice, bank voles, and cotton rats, indicate that males are more commonly infected than females (4, 8, 11, 19, 20, 27). Because these studies used serology to determine hantavirus infection, sex differences in infection could reflect either a lack of infection or the absence of sustained antibody production in females. Experimental inoculation of female rodents with hantavirus, however, illustrates that females produce long-lasting, detectable antibody (22). Alternatively, sex differences in hantavirus prevalence may reflect differences in endocrine-immune interactions (15). The extent to which sex steroids affect immune responses against hantavirus infection has not been examined.

In contrast to other rodent species, sex differences in hantavirus prevalence have not been reported consistently among natural populations of Norway rats. Among adult rats, however, males (90%) tend to be infected with Seoul virus more often than females (75%) (7, 10). Seoul virus is hypothesized to be transmitted via wounding, and adult male rats are more likely to be wounded than either females or juvenile males (10). Thus, sex differences in hantavirus prevalence may reflect complex interactions between behavior and physiology. The first goal of this study was to control for sex differences in exposure and determine whether males were more susceptible to hantavirus infection than females. At 70 to 80 days of age, 5 to 10 male and 5 to 10 female Long Evans rats (*Rattus norvegicus*) were inoculated with either 10^{-4}, 10^{-3}, 10^{-2}, 10^{2}, 10^{4}, or 10^{6} PFU of Seoul virus (strain SR-11) suspended in 0.2 ml of Eagle minimum essential medium (with Earle's salts; Meditach Cellgro, Va.). Seoul virus was obtained from the U.S. Army Medical Research Institute of Infectious Diseases (Ft. Detrick, Md.), where the virus was isolated from neonatal rat brains and

passaged four times in Vero E6 cells. Blood samples were obtained from each animal prior to infection and then 10, 20, 30, and 40 days postinoculation under anesthesia with methoxyflurane vapors (Metofane; Schering Plough, Union, N.J.).

Plasma was used to detect anti-Seoul virus immunoglobulin G (IgG) using an enzyme-linked immunosorbent assay in which microtiter plates were coated overnight at 4°C with gamma-irradiated Vero E6 cells infected with Seoul virus or gamma-irradiated uninfected Vero E6 cells diluted 1:500 in carbonate buffer. Thawed plasma samples, as well as positive control samples (i.e., pooled plasma from rats previously determined to have anti-Seoul virus IgG) and negative control samples (i.e., pooled plasma from Seoul virus-naive rats), were diluted 1:100 in phosphate-buffered saline (PBS)–Tween (PBS-T) with 2% fetal bovine serum and added in duplicate to antigen-coated wells containing either infected or uninfected Vero E6 cells. The plates were sealed, incubated at 37°C for 1 h, and washed with PBS-T, and secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, Md.; alkaline phosphatase-conjugated anti-rat IgG [heavy plus light chains], horseradish peroxidase-conjugated anti-rat IgG1, or horseradish peroxidase-conjugated anti-rat IgG2a diluted 1:400 in PBS with 2% fetal bovine serum) was added. The plates were resealed, incubated for 1 h at 37°C, and washed with PBS-T, and substrate buffer (0.5 mg of *p*-nitrophenyl phosphate per ml diluted in diethanolamine substrate buffer for alkaline phosphatase reactions or tetramethylbenzidine for horseradish peroxidase reactions) was added to each well. Plates were protected from light during the enzyme-substrate reaction, which was terminated after 30 to 45 min by adding 1.5 M NaOH to each well for alkaline phosphatase reactions or 2 N H_2SO_4 to each well for horseradish peroxidase reactions. The optical density (OD) was measured at 405 nm for alkaline phosphatase reactions and 450 nm for horseradish peroxidase reactions, and the average OD for each set of uninfected Vero E6 duplicates was subtracted from the average OD for each set of infected Vero E6 duplicates. Samples were considered positive if the average adjusted OD was ≥0.100. To minimize intra- and interplate variability, the average adjusted OD for each sample

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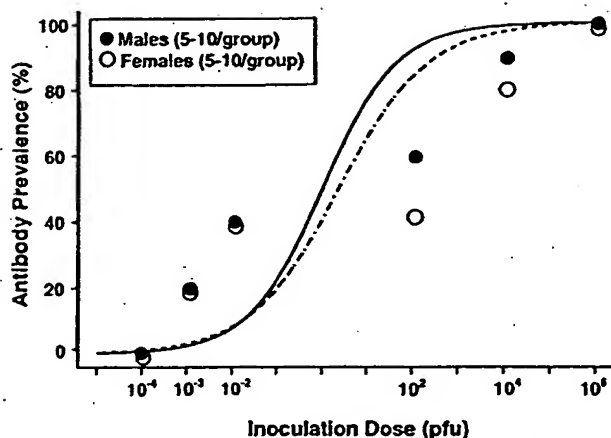
FIG. 1. Antibody prevalence among intact male and female rats inoculated with either 10^{-4}, 10^{-3}, 10^{-2}, 10^{2}, 10^{4}, or 10^{6} PFU of Seoul virus. Data are presented as percentages of individuals producing detectable antibody (i.e., adjusted average OD $\geq$ 0.100) against Seoul virus by day 40 postinoculation, with the fitted logistic regression curves for both males (solid line) and females (dashed line) included. Equal percentages of males and females seroconverted in response to each dose of Seoul virus ($P > 0.05$ in each case).

was expressed as a percentage of its plate-positive control OD for statistical analyses (9).

Antibody prevalence (i.e., the number of animals with detectable anti-Seoul virus IgG) by day 40 postinoculation was compared between males and females using chi-square analyses. Antibody prevalence was assessed 40 days after inoculation because previous studies illustrate that hantavirus-specific antibody is detectable 15 to 30 days postinoculation (7, 14, 22). Antibody prevalence did not differ between males and females at any of the six doses of Seoul virus ($P > 0.05$). Logistic regression was used to compare the infective-dose (ID) curves and estimate the 50% ID (ID_{50}). The ID_{50} did not differ significantly between males (mean $\pm$ standard deviation, 1.1 $\pm$ 2.0 PFU) and females (7.6 $\pm$ 2.0 PFU) (Fig. 1).

Although the prevalence of males and females that became infected did not differ, studies of other viral infections suggest that patterns of immune responses differ between the sexes and are mediated by sex steroid hormones (1, 15, 29). Thus, males and females may differ because testosterone suppresses and estradiol enhances several aspects of immune function (1, 15, 17, 24, 26, 29). The second aim of this study was to examine whether adult sex steroid hormone concentrations influence immune responses and virus shedding following hantavirus infection. Immunologically, patterns of helper T (Th) cell responses (i.e., Th1 or Th2) differ between males and females, with males exhibiting elevated Th1 responses (i.e., elevated gamma interferon, interleukin-2 [IL-2], and IgG2a levels) and females exhibiting increased Th2 responses (i.e., higher IL-4, IL-5, IL-6, and IL-10 levels) (5, 12, 13). Treatment of males with estradiol and females with testosterone prior to infection with pathogens, such as coxsackievirus, reverses the Th responses, suggesting that hormones can modify immune responses to virus infection (12, 13). To determine whether adult sex steroid hormone concentrations influence immune responses and virus shedding following hantavirus infection, at 70 to 80 days of age 20 male and 20 female rats were bilaterally gonadectomized under ketamine (80 mg/kg of body mass)-xylazine (6 mg/kg) anesthesia (Phoenix Pharmaceutical, St. Joseph, Mo.) and given 2 weeks to recover from surgery. After recovery, 10 castrated males were each subcutaneously implanted with a 30-mm Silastic capsule (inside diameter [i.d.] =

1.47 mm, outside diameter [o.d.] = 1.96 mm) containing 20 mm of testosterone propionate (Sigma, St. Louis, Mo.). The remaining 10 castrated males, as well as 10 intact males, were each implanted with an empty capsule of equal length. Ten ovariectomized females were each subcutaneously implanted with a 15-mm Silastic capsule (i.d. = 1.47 mm, o.d. = 1.96 mm) containing 10 mm of estradiol benzoate (Sigma). The remaining 10 ovariectomized females and 9 intact females were each implanted with an empty Silastic capsule of equal length. Silastic capsule length was based on previous reports that these hormone doses (i.e., the length of the Silastic capsule) are sufficient to maintain physiological testosterone and estradiol concentrations in male and female rats, respectively (25). At the time the Silastic capsules were implanted, all animals received an intraperitoneal inoculation of 10^4 PFU of Seoul virus (strain SR-11) suspended in 0.2 ml of Eagle minimum essential medium (i.e., the ID_{90} from the first experiment). Blood, saliva, and fecal samples were then obtained from each animal on days 0, 10, 15, 20, 30, and 40 postinoculation under anesthesia with methoxyflurane vapors. Saliva samples were collected from anesthetized rats after injecting them intraperitoneally with 2.5 mg of pilocarpine HCl (Sigma) per kg of body mass suspended in 0.9% sterile saline (6). After samples were collected on day 40 postinoculation, animals were killed and seminal vesicles were removed from the males and weighed as an index of long-term testosterone concentrations. All procedures described in this paper were approved by the Johns Hopkins Animal Care and Use Committee (protocol number RA98H536) and the Johns Hopkins Office of Health, Safety, and Environment (registration number A9902030102).

Relative seminal vesicle weights (i.e., corrected for body mass) were higher among intact males (0.282 $\pm$ 0.13 g) and castrated males treated with testosterone (0.326 $\pm$ 0.12 g) than among castrated males (0.095 $\pm$ 0.06 g) [$F(2, 29) = 12.75, P < 0.05$]. Plasma testosterone concentrations in males and estradiol concentrations in females were assayed by radioimmunoassay using the manufacturer's protocols (ICN Biochemicals, Inc., Carson, Calif.). Testosterone concentrations were higher for intact males and castrated males treated with testosterone than for castrated male rats; castrated males treated with testosterone also had higher testosterone concentrations than intact males on days 10, 15, 20, and 30, but not on day 40, postinoculation [$F(10, 179) = 19.30, P < 0.05$] (Table 1). Plasma estradiol concentrations were higher for intact females and ovariectomized females treated with estradiol than for ovariectomized females 10, 15, 20, 30, and 40 days postinoculation; ovariectomized females treated with estradiol also had higher estradiol concentrations than intact females on days 10, 15, 20, 30, and 40 postinoculation [$F(10, 173) = 10.29, P < 0.05$] (Table 1).

Manipulation of testosterone concentrations in males and estradiol concentrations in females did not affect production of antibody against Seoul virus ($P > 0.05$). Overall, males had higher anti-Seoul virus IgG responses than females on days 20, 30, and 40 postinoculation, regardless of hormone treatment [$F(5, 353) = 18.72, P < 0.05$] (Table 2). Male rats also had higher anti-Seoul virus IgG2a responses than females on days 30 and 40 postinoculation despite hormone manipulation [$F(5, 353) = 7.81, P < 0.05$] (Fig. 2A). In contrast, females tended to show higher IgG1 responses than males on days 30 and 40 postinoculation, though this did not reach statistical significance ($P > 0.05$) (Fig. 2B).

Viral RNA was identified using nested reverse transcription-PCR (RT-PCR), and the presence of virus in saliva and feces was used to determine whether virus was shed. Viral RNA was isolated using a guanidine isothiocyanate procedure (3). For

TABLE 1. Sex steroid hormone concentrations[a]

Hormone and group	Hormone concn (mean ± SE) on day postinoculation[b]					
	0	10	15	20	30	40
Testosterone						
Intact males	0.69 ± 0.17*	0.84 ± 0.17*	1.13 ± 0.36*	0.92 ± 0.25*	0.77 ± 0.19*	0.70 ± 0.13*
Castrated males	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T-treated males	0.00 ± 0.00	8.24 ± 0.74*†	6.28 ± 0.91*†	6.62 ± 1.18*†	2.73 ± 0.42*†	0.71 ± 0.28*
Estradiol						
Intact females	25.8 ± 6.81*	27.0 ± 5.57*	20.8 ± 8.39*	25.9 ± 7.78*	38.2 ± 10.1*	55.2 ± 10.2*
Ovx females	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
E$_2$-treated females	0.00 ± 0.00	166.6 ± 20.6*†	123.1 ± 21.9*†	87.5 ± 8.9*†	162.3 ± 18.8*†	109.7 ± 19.3*†

[a] Sex steroid hormone concentrations in males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E$_2$]-filled capsules).

[b] Testosterone levels are in nanograms per milliliter, and estradiol levels are in picograms per milliliter. An asterisk indicates that intact and hormone-treated animals had higher hormone concentrations than their gonadectomized counterparts on the corresponding day, based on an analysis of variance ($P < 0.05$). A dagger indicates that hormone-treated animals had higher sex steroid concentrations than their intact counterparts on the corresponding day, based on an analysis of variance ($P < 0.05$).

RNA isolation from saliva, samples were collected from each rat and added to Trizol LS reagent (Life Technologies, Rockville, Md.) at a 3:1 ratio, with RNase-free glycogen (10 μg) added as a carrier. For RNA isolation from feces, approximately 100 mg of feces was homogenized in Tris-EDTA buffer (pH 8.0) and centrifuged at 12,000 × g for 10 min at 4°C; supernatants were collected, incubated with proteinase K (50 μg/ml; Life Technologies) and 0.5% sodium dodecyl sulfate at 50°C for 30 min to digest proteins, and then added to Trizol LS at a 3:1 ratio. To separate, precipitate, and resuspend viral RNA, the manufacturer's protocol was used (Trizol LS; Life Technologies).

For RT-PCR, a 280-bp nucleotide sequence of the SR-11 small (S) genome was amplified using two 20-bp primers, HTN-S4 (5′ GATAGGTGTCCACCAACATG 3′) and HTN-S6 (5′ AGCTCTGGATCCATGTCATC 3′), that amplified positions 979 through 1259 (3). The DNA fragment obtained from the RT-PCR was further amplified using primers HTN-S3 (5′ GCCTTCTTTTCTATACTTCAGG 3′) and HTN-S5 (5′ CCAGGCAACCATAAACATAAC 3′), designed to amplify a 176-bp nucleotide sequence (positions 1031 through 1207). First-strand cDNA was prepared using the GeneAmp RNA PCR kit protocol (Perkin-Elmer, Branchburg, N.J.), incubated in a DNA thermocycler (Techne Genius) at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min, and then held at 4°C. The reaction mixture contained 5 mM MgCl$_2$, 1 mM deoxynucleoside triphosphates, 1 U of RNase inhibitor, and 2.5 U of murine leukemia virus reverse transcriptase. The positive control was SR-11 RNA isolated

from virus stock, and the negative control was diethyl pyrocarbonate water that was included in the cDNA syntheses and primary and secondary amplifications.

The 280-bp sequence was amplified in a 100-μl reaction mixture containing 20 μl of the cDNA, 0.3 μM HTN-S6 primer, and 2.5 U of polymerase (AmpliTaq; Perkin-Elmer). Reactions were amplified for one cycle at 94°C for 3 min and 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 60 s, followed by 10 min at 72°C. The nested 176-bp sequence was amplified in a 100-μl reaction mixture containing 2 μl of the product of the first DNA amplification, 20 μM HTN-S3 primer, 20 μM HTN-S5 primer, 10 mM MgCl$_2$, 1 mM deoxynucleoside triphosphates, and 2.5 U of polymerase. Nested-PCR products were amplified using the same cycle series as was used for the primary amplification. The PCR products were electrophoresed on a 4% gel (3% NuSieve plus 1% SeaKem; FMC Bioproducts, Rockland, Maine), stained with ethidium bromide, and examined for bands of the appropriate size. Randomly selected positive PCR products from saliva and fecal samples from males and females, as well as positive and negative control products, were purified using QIAquick (Qiagen, Valencia, Calif.) and sequenced.

Virus shedding in saliva and feces was not altered by hormone manipulation ($P > 0.05$) (Table 3). Overall, more males shed virus in saliva than females 10 days ($\chi^2 = 3.82$, $df = 1$, $P = 0.051$) and 30 days ($\chi^2 = 8.19$, $df = 1$, $P < 0.05$) after inoculation with Seoul virus (Table 3). The prevalence of Seoul virus in feces also differed between males and females on day 30 postinoculation; more males shed virus in feces than females

TABLE 2. Plasma anti-Seoul virus IgG responses[a]

Group	Anti-Seoul virus IgG response (mean ± SE) on day postinoculation[b]					
	0	10	15	20	30	40
Intact males	0.8 ± 0.6	4.9 ± 3.0	84.0 ± 22.0	106.0 ± 19.0*	332.0 ± 47.0*	342.1 ± 56.0*
Castrated males	1.0 ± 0.7	1.0 ± 1.0	82.0 ± 21.0	106.0 ± 27.0*	280.0 ± 71.0*	387.3 ± 84.0*
T-treated males	1.0 ± 0.7	2.0 ± 0.9	33.0 ± 10.0	108.0 ± 14.0*	314.0 ± 41.0*	426.7 ± 43.0*
Intact females	3.0 ± 1.0	9.0 ± 4.0	36.0 ± 10.0	60.0 ± 14.0	189.0 ± 55.0	219.6 ± 63.0
Ovx females	2.0 ± 0.8	4.0 ± 2.0	7.0 ± 3.0	54.0 ± 16.0	187.0 ± 56.0	209.2 ± 53.0
E$_2$-treated females	3.0 ± 1.0	8.0 ± 2.0	19.0 ± 6.0	39.0 ± 8.0	178.0 ± 42.0	209.1 ± 39.0

[a] Plasma anti-Seoul virus IgG responses in males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E$_2$]-filled capsules).

[b] Data are presented as IgG units, in which the mean OD of each test sample was divided by the OD of the positive control sample run on the same microtiter plate. An asterisk indicates that males had higher IgG responses than females, regardless of hormone manipulation, based on an analysis of variance ($P < 0.05$).

A

B

FIG. 2. (A) Plasma anti-Seoul virus IgG2a responses (mean ± standard error) in male and female rats. (B) Plasma anti-Seoul virus IgG1 responses (mean ± standard error) in male and female rats. Blood samples were collected 0, 10, 15, 20, 30, and 40 days following inoculation with Seoul virus. For calculation of IgG2a or IgG1 units, the mean OD of each test sample was divided by the OD of the positive control sample run on the same microtiter plate. Because neither gonadectomy nor hormone replacement had an effect on antibody production, responses from the different treatments groups were collapsed and graphed together. An asterisk indicates that males had higher IgG2a responses than females ($P < 0.05$).

($\chi^2 = 6.88$, $df = 1$, $P < 0.05$) (Table 3). In general, males shed virus in saliva and feces more consistently than females, regardless of hormone manipulation (Table 3). The PCR product obtained from saliva and feces of males and females was sequenced and verified as Seoul virus DNA.

Sex differences in the prevalence of hantavirus infection have been observed in several natural rodent populations, including deer mice, brush mice, harvest mice, bank voles, and cotton rats (4, 8, 11, 19, 20, 27). In each case, males are infected more often than females. Field studies of Norway rats suggest that sex differences in hantavirus prevalence reflect sex differences in behaviors, like aggression, that increase the likelihood of males being infected (10). High circulating testosterone concentrations increase the probability of engaging in aggressive encounters in several vertebrate species (21). In addition to modulating aggression, sex steroid hormones can

affect immune responses against infection. Studies of viral infections, such as coxsackievirus, suggest that sex differences in both the prevalence and intensity of infection are due to differences in endocrine-immune interactions (12, 13).

Despite the known effects of sex steroids on infection, in the present study, manipulation of adult sex steroids had no effect on immune responses or virus shedding following exposure to Seoul virus. Specifically, males had higher antibody responses and shed virus longer than females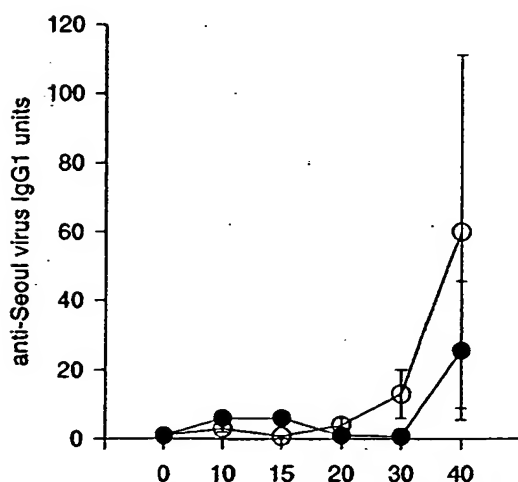, regardless of adult hormone manipulation. Sex steroid hormones affect physiology and behavior at two distinct times during ontogeny (2, 16, 23). During perinatal development, sex steroids cause sex differences in the differentiation or organization of central and peripheral structures. In adulthood, exposure to sex steroids serves to activate preexisting hormonal circuits. The data from the present study may suggest that sex steroid hormones are not involved in hantavirus infection. Alternatively, these data may illustrate that manipulation of activational sex steroids does not alter responses to infection because the hormonal circuitry was organized earlier during development. If sex steroids organize adult responses to infection, then manipulation of neonatal sex steroids should alter adult responses to hantavirus infection.

Regardless of hormone manipulation, males had higher anti-Seoul virus IgG2a responses than females. Recent data from our laboratory indicate that following Seoul virus inoculation, males have elevated IL-2 and gamma interferon concentrations and females have elevated IL-4 responses (S. L. Klein and G. E. Glass, unpublished data). Taken together, these data suggest that males may have higher Th1 responses to hantavirus infection than females. Studies of other viral infections in rodents suggest that females typically have higher Th2 re-

TABLE 3. Virus shedding[a]

Sample and group	No. of virus-shedding rats/total on day postinoculation[b]				
	10	15	20	30	40
Saliva samples					
Intact males	6/11	7/10	6/11	6/11	6/11
Castrated males	4/9	4/9	6/9	5/9	8/9
T-treated males	9/10	7/10	4/10	6/10	7/10
Total males	19/30*	18/29	16/30	17/30*	21/30
Intact females	3/9	6/9	5/9	2/9	2/9
Ovx females	4/10	7/10	2/10	2/10	6/10
E₂-treated females	3/10	10/10	3/10	1/10	6/10
Total females	10/29	23/29	11/29	5/29	14/29
Fecal samples					
Intact males	5/11	4/11	4/11	5/11	1/11
Castrated males	6/9	5/9	7/9	4/8	1/9
T-treated males	4/10	6/10	7/10	7/9	1/10
Total males	15/30	15/30	18/30	16/29*	3/30
Intact females	7/9	4/9	4/9	1/8	0/9
Ovx females	9/10	4/10	6/10	2/10	2/10
E₂-treated females	6/9	5/10	8/10	2/10	1/10
Total females	22/28	13/29	18/29	5/28	3/29

[a] Virus shedding in saliva and feces from males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E₂]-filled capsules).

[b] An asterisk indicates that more males shed virus than females on the respective day postinoculation, based on chi-square analyses ($P < 0.05$).

sponses than males and that this is due, in part, to the effects of estrogens on cytokine production (12). In the present study, females tended to produce higher IgG1 responses than males. In contrast to estrogens, androgens promote differentiation of CD4$^+$ T cells to a Th1 phenotype (12). In the present study, however, castrated and intact males had similar IgG2a responses, suggesting that increased Th1 responses are not contingent on the direct effects of androgens.

High antibody responses in males may indicate that males have more efficient immune responses against infection than females. This outcome seems unlikely given the rapid increase and long duration of virus shedding in males compared to females. Alternatively, males may have higher antibody responses than females because virus replication is increased in males. Higher Th1 responses are associated with increased susceptibility to infections caused by coxsackievirus and Sindbis virus in mice (12, 28). Although quantitative analyses were not conducted, males shed Seoul virus longer than females, suggesting that higher Th1 responses among males may be a consequence of increased virus replication.

In summary, although males and females are equally susceptible to infection with Seoul virus, males shed virus longer and produce higher Th1 responses against Seoul virus than females. Increased virus shedding among males may explain why males are more likely to acquire Seoul virus infection following aggressive encounters among natural populations of Norway rats (10). In the present study, manipulation of adult sex steroid hormones did not alter immune responses or virus shedding following inoculation with Seoul virus. Although sex steroid hormones may not mediate sex differences in response to hantavirus infection, sex differences in infection among adults may be altered by sex steroids earlier during development. Alternatively, sex differences in infection may reflect other neuroendocrine changes, such as differences in glucocorticoids, that may affect responses to Seoul virus infection.

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EXHIBIT 4

Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions

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An amino acid sequence encodes a message that determines the shape and function of a protein. This message is highly degenerate in that many different sequences can code for proteins with essentially the same structure and activity. Comparison of different sequences with similar messages can reveal key features of the code and improve understanding of how a protein folds and how it performs its function.

THE GENOME IS MANIFEST LARGELY IN THE SET OF PROteins that it encodes. It is the ability of these proteins to fold into unique three-dimensional structures that allows them to function and carry out the instructions of the genome. Thus, comprehending the rules that relate amino acid sequence to structure is fundamental to an understanding of biological processes. Because an amino acid sequence contains all of the information necessary to determine the structure of a protein (1), it should be possible to predict structure from sequence, and subsequently to infer detailed aspects of function from the structure. However, both problems are extremely complex, and it seems unlikely that either will be solved in an exact manner in the near future. It may be possible to obtain approximate solutions by using experimental data to simplify the problem. In this article, we describe how an analysis of allowed amino acid substitutions in proteins can be used to reduce the complexity of sequences and reveal important aspects of structure and function.

Methods for Studying Tolerance to Sequence Variation

There are two main approaches to studying the tolerance of an amino acid sequence to change. The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. This method has been extremely powerful for proteins such as the globins or cytochromes, for which sequences from many different species are known (2–7). The second approach uses genetic methods to introduce amino acid changes at

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specific positions in a cloned gene and uses selections or screens to identify functional sequences. This approach has been used to great advantage for proteins that can be expressed in bacteria or yeast, where the appropriate genetic manipulations are possible (3, 8–11). The end results of both methods are lists of active sequences that can be compared and analyzed to identify sequence features that are essential for folding or function. If a particular property of a side chain, such as charge or size, is important at a given position, only side chains that have the required property will be allowed. Conversely, if the chemical identity of the side chain is unimportant, then many different substitutions will be permitted.

Studies in which these methods were used have revealed that proteins are surprisingly tolerant of amino acid substitutions (2–4, 11). For example, in studying the effects of approximately 1500 single amino acid substitutions at 142 positions in lac repressor, Miller and co-workers found that about one-half of all substitutions were phenotypically silent (11). At some positions, many different, nonconservative substitutions were allowed. Such residue positions play little or no role in structure and function. At other positions, no substitutions or only conservative substitutions were allowed. These residues are the most important for lac repressor activity.

What roles do invariant and conserved side chains play in proteins? Residues that are directly involved in protein functions such as binding or catalysis will certainly be among the most conserved. For example, replacing the Asp in the catalytic triad of trypsin with Asn results in a 10^4-fold reduction in activity (12). A similar loss of activity occurs in λ repressor when a DNA binding residue is changed from Asn to Asp (13). To carry out their function, however, these catalytic residues and binding residues must be precisely oriented in three dimensions. Consequently, mutations in residues that are required for structure formation or stability can also have dramatic effects on activity (10, 14–16). Hence, many of the residues that are conserved in sets of related sequences play structural roles.

Substitutions at Surface and Buried Positions

In their initial comparisons of the globin sequences, Perutz and co-workers found that most buried residues require nonpolar side chains, whereas few features of surface side chains are generally conserved (6). Similar results have been seen for a number of protein families (2, 4, 5, 7, 17, 18). An example of the sequence tolerance at surface versus buried sites can be seen in Fig. 1, which shows the allowed substitutions in λ repressor at residue positions that are near the dimer interface but distant from the DNA binding surface of the protein (9). These substitutions were identified by a functional

Fig. 1. (A) Amino acid substitutions allowed in a short region of λ repressor. The wild-type sequence is shown along the center line. The allowed substitutions shown above each position were identified by randomly mutating one to three codons at a time by using a cassette method and applying a functional selection (9). (B) The fractional solvent accessibility (42) of the wild-type side chain in the protein dimer (43) relative to the same atoms in an Ala-X-Ala model tripeptide.
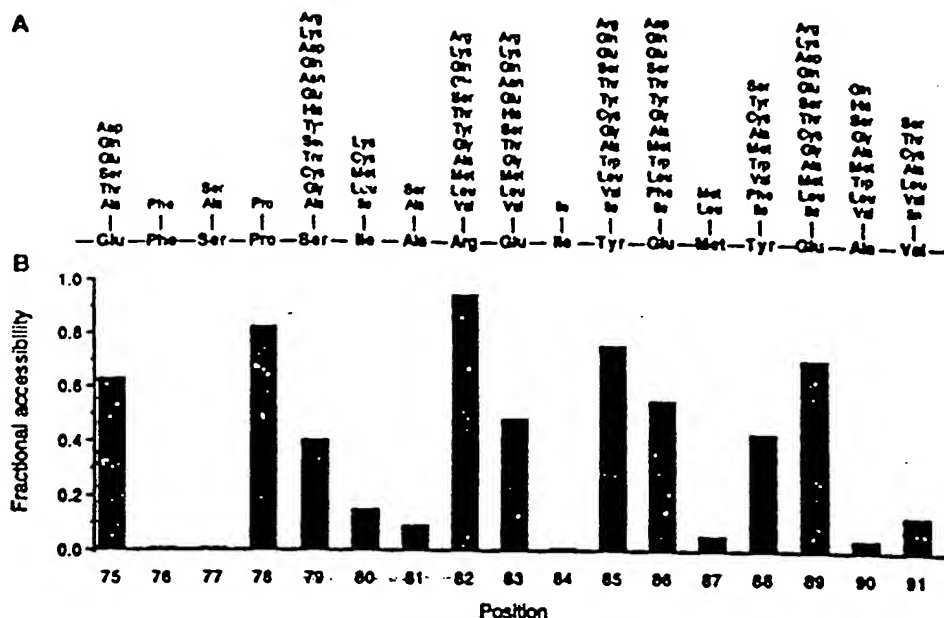
selection after cassette mutagenesis. A histogram of side chain solvent accessibility in the crystal structure of the dimer is also shown in Fig. 1. At six positions, only the wild-type residue or relatively conservative substitutions are allowed. Five of these positions are buried in the protein. In contrast, most of the highly exposed positions tolerate a wide range of chemically different side chains, including hydrophilic and hydrophobic residues. Hence, it seems that most of the structural information in this region of the protein is carried by the residues that are solvent inaccessible.

Constraints on Core Sequences

Because core residue positions appear to be extremely important for protein folding or stability, we must understand the factors that dictate whether a given core sequence will be acceptable. In general, only hydrophobic or neutral residues are tolerated at buried sites in proteins, undoubtedly because of the large favorable contribution of the hydrophobic effect to protein stability (19). For example, Fig. 2 shows the results of genetic studies used to investigate the substitutions allowed at residue positions that form the hydrophobic core of the NH2-terminal domain of λ repressor (20). The acceptable core sequences are composed almost exclusively of Ala, Cys, Thr, Val, Ile, Leu, Met, and Phe. The acceptability of many different residues at each core position presumably reflects the fact that the hydrophobic effect, unlike hydrogen bonding, does not depend on specific residue pairings. Although it is possible to imagine a hypothetical core structure that is stabilized exclusively by residues forming hydrogen bonds and salt bridges, such a core would probably be difficult to construct because hydrogen bonds require pairing of donors and acceptors in an exact geometry. Thus the repertoire of possible structures that use a polar core would probably be extremely limited (21). Polar and charged residues are occasionally found in the cores of proteins, but only at positions where their hydrogen bonding needs can be satisfied (22).

The cores of most proteins are quite closely-packed (23), but some volume changes are acceptable. In λ repressor, the overall core volume of acceptable sequences can vary by about 10%. Changes at individual sites, however, can be considerably larger. For example, as shown in Fig. 2, both Phe and Ala are allowed at the same core position in the appropriate sequence contexts. Large volume changes at individual buried sites have also been observed in

phylogenetic studies, where it has been noted that the size decreases and increases at interacting residues are not necessarily related in a simple complementary fashion (5, 7, 17). Rather, local volume changes are accommodated by conformational changes in nearby side chains and by a variety of backbone movements.

The Informational Importance of the Core

With occasional exceptions, the core must remain hydrophobic and maintain a reasonable packing density. However, since the core is composed of side chains that can assume only a limited number of conformations (24), efficient packing must be maintained without steric clashes. How important are hydrophobicity, volume, and steric complementarity in determining whether a given sequence can form an acceptable core? Each factor is essential in a physical sense, as a stable core is probably unable to tolerate unsatisfied hydrogen bonding groups, large holes, or steric overlaps (25). However, in an informational sense, these factors are not equivalent. For example, in experiments in which three core residues of λ repressor were mutated simultaneously, volume was a relatively unimportant informational constraint because three-quarters of all possible combinations of the 20 naturally occurring amino acids had volumes within the range tolerated in the core, and yet most of these sequences were unacceptable (20). In contrast, of the sequences that contained only

Fig. 2. Amino acid substitutions allowed in the core of λ repressor. The wild-type side chains are shown pictorially in the approximate orientation seen in the crystal structure (43). The lists of allowed substitutions at each position are shown below the wild-type side chains. These substitutions were identified by randomly mutating one to four residues at a time by using a cassette method and applying a functional selection (20). Not all substitutions are allowed in every sequence background.

40	36	47	65	51	57	18
Ala	Ala	Ala	Ala	Cys	Cys	Ala
Cys	Cys	Cys	Ser	Val	Pro	Cys
Val	Thr	Thr	Cys	Ile	Val	Val
Ile	Val	Val	Thr	Leu	Ile	Ile
Leu	Ile	Ile	Val	Met	Leu	Leu
Met	Leu	Leu	Ile	Phe	Met	Met
Phe	Met	Met	Ile		Phe	Phe
	Phe	Phe	Leu			
			Met			
			Phe			
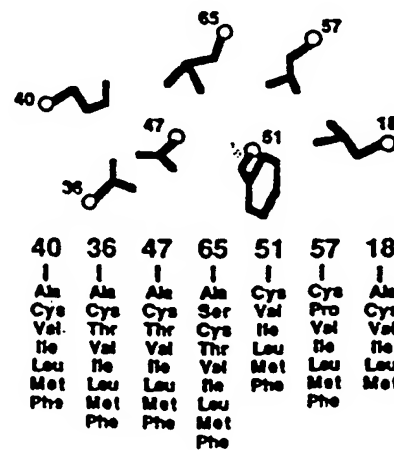
the appropriate hydrophobic residues, a significant fraction were acceptable. Hence, the hydrophobicity of a sequence contains more information about its potential acceptability in the core than does the total side chain volume. Steric compatibility was intermediate between volume and hydrophobicity in informational importance.

The Informational Importance of Surface Sites

We have noted that many surface sites can tolerate a wide variety of side chains, including hydrophilic and hydrophobic residues. This result might be taken to indicate that surface positions contain little structural information. However, Bashford et al., in an extensive analysis of globin sequences (4), found a strong bias against large hydrophobic residues at many surface positions. At one level, this may reflect constraints imposed by protein solubility, because large patches of hydrophobic surface residues would presumably lead to aggregation. At a more fundamental level, protein folding requires a partitioning between surface and buried positions. Consequently, to achieve a unique native state without significant competition from other conformations, it may be important that some sites have a decided preference for exterior rather than interior positions. As a result, many surface sites can accept hydrophobic residues individually, but the surface as a whole can probably tolerate only a moderate number of hydrophobic side chains.

Identification of Residue Roles from Sets of Sequences

Often, a protein of interest is a member of a family of related sequences. What can we infer from the pattern of allowed substitutions at positions in sets of aligned sequences generated by genetic or phylogenetic methods? Residue positions that can accept a number of different side chains, including charged and highly polar residues, are almost certain to be on the protein surface. Residue positions that remain hydrophobic, whether variable or not, are likely to be buried within the structure. In Fig. 3, those residue positions in λ repressor that can accept hydrophilic side chains are shown in orange and those that cannot accept hydrophilic side chains are shown in green. The obligate hydrophobic positions define the core of the structure, whereas positions that can accept hydrophilic side chains define the surface.

Functionally important residues should be conserved in sets of active sequences, but it is not possible to decide whether a side chain is functionally or structurally important just because it is invariant or conserved. To make this distinction requires an independent assay of protein folding. The ability of a mutant protein to maintain a stably folded structure can often be measured by biophysical techniques, by susceptibility to intracellular proteolysis (26), or by binding to antibodies specific for the native structure (27, 28). In the latter cases, it is possible to screen proteins in mutated clones for the ability to fold even if these proteins are inactive. Sets of sequences that allow formation of a stable structure can then be compared to the sets that allow both folding and function, with the active site or binding residues being those that are variable in the set of stable proteins but invariant in the set of functional proteins. The DNA-binding residues of Arc repressor were identified by this method (8). The receptor-binding residues of human growth hormone were also identified by comparing the stabilities and activities of a set of mutant sequences (28). However, in this case, the mutants were generated as hybrid sequences between growth hormone and related hormones with different binding specificities.

Implications for Structure Prediction

At present, the only reliable method for predicting a low-resolution tertiary structure of a new protein is by identifying sequence similarity to a protein whose structure is already known (29, 30). However, it is often difficult to align sequences as the level of sequence similarity decreases, and it is sometimes impossible to detect statistically significant sequence similarity between distantly related proteins. Because the number of known sequences is far greater than the number of known structures, it would be advantageous to increase the reach of the available structural information by improving methods for detecting distant sequence relations and for subsequently aligning these sequences based on structural principles. In a normal homology search, the sequence database is scanned with a single test sequence, and every residue must be weighted equally. However, some residues are more important than others and should be weighted accordingly. Moreover, certain regions of the protein are more likely to contain gaps than others. Both kinds of information can be obtained from sequence sets, and several techniques have
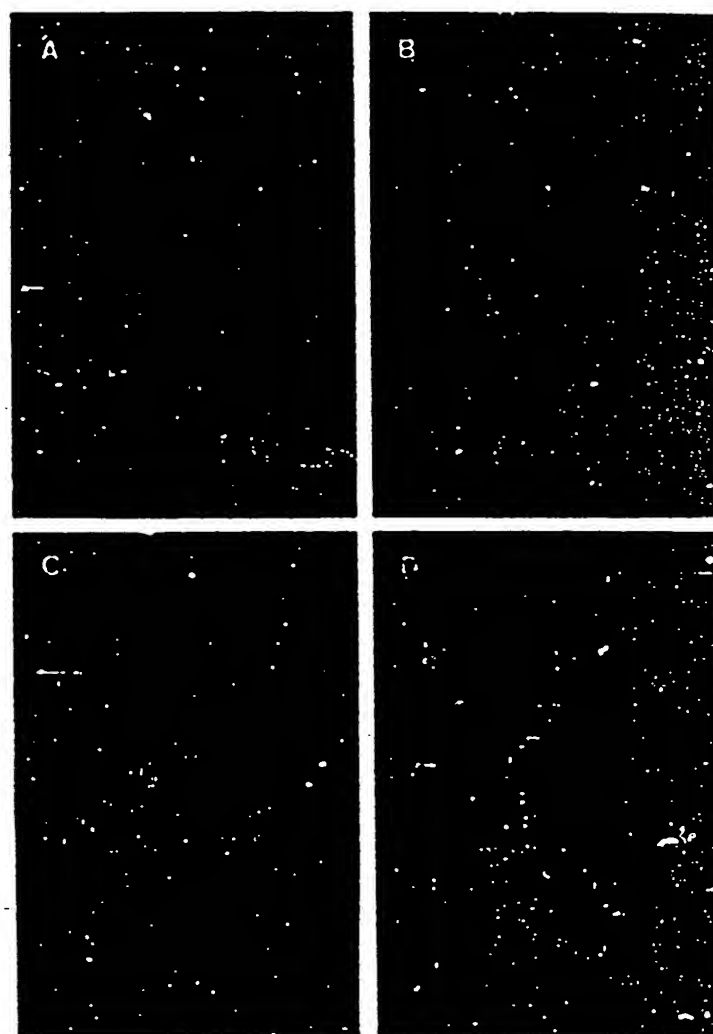
Fig. 3. Tolerance of positions in the NH$_2$-terminal domain of λ repressor to hydrophilic side chains. The complex (43) of the repressor dimer (blue) and operator DNA (white) is shown. In (A), positions that can tolerate hydrophilic side chains are shown in orange. The same side chains are shown in (B) without the remaining protein atoms. In (C), positions that require hydrophobic or neutral side chains are shown in green. These side chains are shown in (D) without the remaining protein atoms. About three-fourths of the 92 side chains in the NH$_2$-terminal domain are included in both (B) and (D). The remaining positions have not been tested. Data are from (9, 14, 20, 27, 44).

been used to combine such information into more appropriately weighted sequence searches and alignments (*31*). These methods were used to align the sequences of retroviral proteases with aspartic proteases, which in turn allowed construction of a three-dimensional model for the protease of human immunodeficiency virus type 1 (*29*). Comparison with the recently determined crystal structure of this protein revealed reasonable agreement in many areas of the predicted structure (*32*).

The structural information at most surface sites is highly degenerate. Except for functionally important residues, exterior positions seem to be important chiefly in maintaining a reasonably polar surface. The information contained in buried residues is also degenerate, the main requirement being that these residues remain hydrophobic. Thus, at its most basic level, the key structural message in an amino acid sequence may reside in its specific pattern of hydrophobic and hydrophilic residues. This is meant in an informational sense. Clearly, the precise structure and stability of a protein depends on a large number of detailed interactions. It is possible, however, that structural prediction at a more primitive level can be accomplished by concentrating on the most basic informational aspects of an amino acid sequence. For example, amphipathic patterns can be extracted from aligned sets of sequences and used, in some cases, to identify secondary structures.

If a region of secondary structure is packed against the hydrophobic core, a pattern of hydrophobic residues reflecting the periodicity of the secondary structure is expected (*33, 34*). These patterns can be obscured in individual sequences by hydrophobic residues on the protein surface. It is rare, however, for a surface position to remain hydrophobic over the course of evolution. Consequently, the amphipathic patterns expected for simple secondary structures can be much clearer in a set of related sequences (*6*). This principle is illustrated in Fig. 4, which shows helical hydrophobic moment plots for the Antennapedia homeodomain sequence (Fig. 4A) and for a composite sequence derived from a set of homologous homeodomain proteins (Fig. 4B) (*35*). The hydrophobic moment is a simple measure of the degree of amphipathic character of a sequence in a given secondary structure (*34*). The amphipathic character of the three α-helical regions in the Antennapedia protein (*36*) is clearly revealed only by the analysis of the combined set of homeodomain sequences. The secondary structure of Arc repressor, a small DNA-binding protein, was recently predicted by a similar method (*8*) and confirmed by nuclear magnetic resonance studies (*37*).

The specific pattern of hydrophobic and hydrophilic residues in an amino acid sequence must limit the number of different structures a given sequence can adopt and may indeed define its overall fold. If this is true, then the arrangement of hydrophobic and hydrophilic residues should be a characteristic feature of a particular fold. Sweet and Eisenberg have shown that the correlation of the pattern of hydrophobicity between two protein sequences is a good criterion for their structural relatedness (*38*). In addition, several studies indicate that patterns of obligatory hydrophobic positions identified from aligned sequences are distinctive features of sequences that adopt the same structure (*4, 29, 38, 39*). Thus, the order of hydrophobic and hydrophilic residues in a sequence may actually be sufficient information to determine the basic folding pattern of a protein sequence.

Although the pattern of sequence hydrophobicity may be a characteristic feature of a particular fold, it is not yet clear how such patterns could be used for prediction of structure de novo. It is important to understand how patterns in sequence space can be related to structures in conformation space. Lau and Dill have approached this problem by studying the properties of simple sequences composed only of H (hydrophobic) and P (polar) groups on two-dimensional lattices (*40*). An example of such a representa-

tion is shown in Fig. 5. Residues adjacent in the sequence must occupy adjacent squares on the lattice, and two residues cannot occupy the same space. Free energies of particular conformations are evaluated with a single term, an attraction of H groups. By considering chains of ten residues, an exhaustive conformational search for all 1024 possible sequences of H and P residues was possible. For longer sequences only a representative fraction of the allowed sequence or conformation space could be explored. The significant results were as follows: (i) not all sequences can fold into a "native" structure and only a few sequences form a unique native structure; (ii) the probability that a sequence will adopt a unique native structure increases with chain length; and (iii) the native states are compact, contain a hydrophobic core surrounded by polar residues, and contain significant secondary structure. Although the gap between these two-dimensional simulations and three-dimensional structures is large, the use of simple rules and sequence representations yields results similar to those expected for real proteins. Three-dimensional lattice methods are also beginning to be developed and evaluated (*41*).

Summary

There is more information in a set of related sequences than in a single sequence. A number of practical applications arise from an analysis of the tolerance of residue positions to change. First, such information permits the evaluation of a residue's importance to the function and stability of a protein. This ability to identify the essential elements of a protein sequence may improve our understanding of the determinants of protein folding and stability as well as protein function. Second, patterns of tolerance to amino acid substitutions of varying hydrophilicity can help to identify residues likely to be buried in a protein structure and those likely to occupy
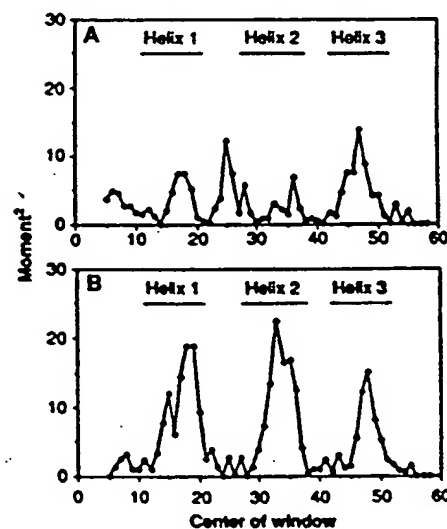
Fig. 4. Helical hydrophobic moments calculated by using (A) the Antennapedia homeodomain sequence or (B) a set of 39 aligned homeodomain sequences (*35*). The bars indicate the extent of the helical regions identified in nuclear magnetic resonance studies of the Antennapedia homeodomain (*36*). To determine hydrophobic moments, residues were assigned to one of three groups: H1 (high hydrophobicity = Trp, Ile, Phe, Leu, Met, Val, or Cys); H2 (medium hydrophobicity = Tyr, Pro, Ala, Thr, His, Gly, or Ser); and H3 (low hydrophobicity = Gln, Asn, Glu, Asp, Lys, or Arg). For the aligned homeodomain sequences, the residues at each position were sorted by their hydrophobicity by using the scale of Fauchere and Pliska (*45*). Arg and Lys were not counted unless no other residue was found at the position, because they contain long aliphatic side chains and can thereby substitute for nonpolar residues at some buried sites. To account for possible sequence errors and rare exceptions, the most hydrophilic residue allowed at each position was discarded unless it was observed twice. The second most hydrophilic residue was then chosen to represent the hydrophobicity of each position. An eight-residue window was used and the vectors projected radially every 100°. The vector magnitudes were assigned a value of 1, 0, or −1 for positions where the hydrophobicity group was H1, H2, or H3, respectively.

P H P P H P H P H H H P P H
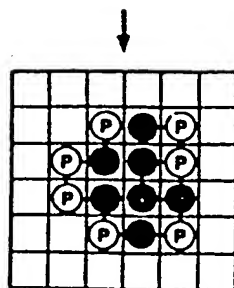
surface positions. The amphipathic patterns that emerge can be used to identify probable regions of secondary structure. Third, incorporating a knowledge of allowed substitutions can improve the ability to detect and align distantly related proteins because the essential residues can be given prominence in the alignment scoring.

As more sequences are determined, it becomes increasingly likely that a protein of interest is a member of a family of related sequences. If this is not the case, it is now possible to use genetic methods to generate lists of allowed amino acid substitutions. Consequently, at least in the short term, it may not be necessary to solve the folding problem for individual protein sequences. Instead, information from sequence sets could be used. Perhaps by simplifying sequence space through the identification of key residues, and by simplifying conformation space as in the lattice methods, it will be possible to develop algorithms to generate a limited number of trial structures. These trial structures could then, in turn, be evaluated by further experiments and more sophisticated energy calculations.

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EXHIBIT 5

From genes to protein structure and function: novel applications of computational approaches in the genomic era

Jeffrey Skolnick and Jacquelyn S. Fetrow

The genome-sequencing projects are providing a detailed 'parts list' of life. A key to comprehending this list is understanding the function of each gene and each protein at various levels. Sequence-based methods for function prediction are inadequate because of the multifunctional nature of proteins. However, just knowing the structure of the protein is also insufficient for prediction of multiple functional sites. Structural descriptors for protein functional sites are crucial for unlocking the secrets in both the sequence and structural-genomics projects.

Genome-sequencing projects are providing a detailed 'parts list' for life. Unfortunately, this list, a portion of which represents the amino acid sequence of all the proteins in a given genome, does not come with an instruction manual. That is, given the genome's sequences, one does not necessarily know straight away which regions encode proteins, which serve a regulatory role and which are responsible for the structure and replication of the DNA itself.

This is not unlike giving a child a list of parts necessary to create a working automobile. Without the necessary expertise, creating the final, working car from just the initial parts list is a nearly impossible task. Similarly, understanding how to create a complete, functioning cell given just the sequence of nucleotides found in an organism's genome is a complex problem.

What is a protein function?

After a genome is sequenced and its complete parts list determined, the next goal is to understand the function(s) of each part, including that of the proteins. What do we mean by protein function, the focus of this article?

Function has many meanings. At one level, the protein could be a globular protein, such as an enzyme, hormone or antibody, or it could be a structural or membrane-bound protein. Another level is its biochemical function, such as the chemical reaction and the substrate specificity of an enzyme. The regulatory molecules or cofactors that bind to a protein are also levels of biochemical function.

At the cellular level, the protein's function would involve its interaction with other macromolecules and the function and cellular location of such complexes. There is also the protein's physiological function; that is, in which metabolic pathway the protein is involved or what physiological role it performs in the organism. Finally, the phenotypic function is the role played by the protein in the total organism, which is observed by deleting or mutating the gene encoding the protein.

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Obviously, the complete characterization of protein function is difficult but efforts are under way at all levels[1–4], including cellular function[5,6]. In this article, however, we focus on identifying the biochemical function of a protein given its sequence, a problem that is amenable to molecular approaches.

Sequence-based approaches to function prediction

The sequence-to-function approach is the most commonly used function-prediction method. This robust field is well developed and, in the interest of space limitations, we will merely present a brief overview.

There are two main flavors of this approach: sequence alignment[7–9]; and sequence-motif methods such as Prosite[10], Blocks[11], Prints[12,13] and Emotif[14]. Both the alignment and the motif methods are powerful but a recent analysis has demonstrated their significant limitations[15], suggesting that these methods will increasingly fail as the protein-sequence databases become more diverse.

An extension of these approaches that combines protein-sequence with structural information has been developed and some successes have been reported[16]. However, this method still applies the structural information in a one-dimensional, 'sequence-like' fashion and fails to take into account the powerful three-dimensional information displayed by protein structures.

In addition, proteins can gain and lose function during evolution and may, indeed, have multiple functions in the cell (Box 1). Sequence-to-function methods cannot specifically identify these complexities. Inaccurate use of sequence-to-function methods has led to significant function-annotation errors in the sequence databases[17].

An alternative approach

An alternative, complementary approach to protein-function prediction uses the sequence-to-structure-to-function paradigm. Here, the goal is to determine the structure of the protein of interest and then to identify the functionally important residues in that structure. Using the chemical structure itself to identify functional sites is more in line with how the protein actually works.

In a sense, this is one long-term goal of 'structural genomics' projects[18,19], which are designed to determine all possible protein folds experimentally, just as genome-sequencing projects are determining all protein sequences[20]. This is in contrast to traditional structural-biology approaches, in which one knows the protein's function first and only then, if the function is sufficiently important, determines its structure.

It is implicitly assumed that having the protein's structure will provide insights into its function, thereby furthering the goals of the human-genome-sequencing project. However, knowing a protein's three-dimensional structure is insufficient to determine its function (Box 2). What we really need to analyse and predict the multifunctional aspects of proteins is a method specifically to recognize active sites and binding regions in these protein structures.

Active-site identification

In order to use a structure-based approach to function prediction, one must identify the key residues responsible for a given biochemical activity. For many years, it has been suggested that the active sites in proteins are better conserved than the overall fold. Taken to the limit, this suggests that one could not only identify distant ancestors with the same global fold and the same activity but also proteins with similar functions but distantly related, or possibly unrelated, global folds.

The validity of this suggestion was demonstrated empirically by Nussinov and co-workers, who showed that the active sites of eukaryotic serine proteases, subtilisins and sulfhydryl proteases exhibit similar structural motifs[21]. Furthermore, in a recent modeling study of Saccharomyces cerevisiae proteins, protein functional sites were found to be more conserved than other parts of the protein models[22]. Similarly, it has been demonstrated that the catalytic triad of the α/β hydrolases is structurally better conserved than other histidine-containing triads[23]. A comparison of the structure of the hydrolase catalytic triad to other histidine-containing triads shows a distinct bimodal distribution, while a similar analysis done with a randomly selected triad shows a unimodal distribution (Fig. 1).

Kasuya and Thornton[24] generalized this example by creating structural analogs of a few Prosite sequence motifs[10]. For the 20 most-frequently occurring Prosite patterns, the associated local structure is quite distinct. These results provide clear evidence that enzyme active sites are indeed more highly conserved than other parts of the protein.

Identifying active sites in experimental structures

Historically, several groups have attempted to identify functional sites in proteins; these efforts were directed at protein engineering or building functional sites in places where they did not previously exist. This has been successfully accomplished for several metal-binding sites[25-33]. However, highly accurate functional-site descriptors of the backbone and side-chain atoms were required, fueling the belief that significant atomic detail is required in site descriptors for function identification.

Highly detailed residue side-chain descriptors of the active sites of serine proteases and related proteins have been used to identify functional sites[3]. The use of these highly detailed motifs has led to the identification of

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Box 1. Proteins are multifunctional

A common protein characteristic that makes functional analysis based only on homology especially difficult is the tendency of proteins to be multifunctional. For instance, lactate dehydrogenase binds NAD, substrate and zinc, and performs a redox reaction. Each of these occurs at different functional sites that are in close proximity and the combination of all four sites creates the fully functional protein.

Other examples of multifunctional proteins are the nucleic-acid-binding proteins. For instance, DNA regulatory proteins often contain a DNA-binding domain, a multimerization domain and additional sites that bind regulatory proteins; a classic example is RecA[59]. The 3C rhinovirus protease exhibits a proteolytic function as well as an RNA-binding function[60,61]. Transcription factors are also complex, multifunctional proteins[62]. It is becoming increasingly important to recognize each of these different functions of gene products of a newly sequenced gene.

The serine–threonine-phosphatase superfamily is a prime example of the difficulties of using standard sequence analysis to recognize the multiple functions found in single proteins. This large protein family is divided into a number of subfamilies, all of which contain an essential phosphatase active site. Subfamilies 1, 2A and 2B exhibit 40% or more sequence identity between them[63]. However, each of these subfamilies is apparently regulated differently in the cell[64-67] and observation suggests that there are different functional sites at which regulation can occur. Because the sequence identity between subfamilies is so high, standard sequence-similarity methods could easily misclassify new sequences as members of the wrong subfamily if the functional sites are not carefully considered, as was recently demonstrated[43].

These are but a few examples of the multifunctionality of proteins. The recognition of this multifunctional nature is of critical importance to the genomics field. Useful functional-annotation methods must consider all of the specific functions in a given protein and will not just provide a general classification of function.

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several novel functional sites in known, high-quality protein structures[3,34]. More automated methods for finding spatial motifs in protein structures have also been described[21,34-40].

Unfortunately, most of these methods require the exact placement of atoms within protein backbones and side chains, and so have not been shown to be relevant to inexact predicted structures. Recently, however, we described the production of fuzzy, inexact descriptors of protein functional sites[15]. As we wish to apply the descriptors to experimental structures as well as to predicted protein models, we used only carbon atoms and side-chain centers-of-mass positions. We call these descriptors 'fuzzy functional forms' (FFFs) and have created them for both the disulfide-oxidoreductase[15,41] and α/β-hydrolase catalytic active sites[23].

The disulfide-oxidoreductase FFF was applied to screen high-resolution structures from the Brookhaven protein database[42]. In a dataset of 364 protein structures, the FFF accurately identified all proteins known to exhibit the disulfide-oxidoreductase active site[15]. In a larger dataset of 1501 proteins, the FFF again accurately identified all proteins with the active site. In addition, it identified another protein, 1fjm, a serine–threonine phosphatase. This result was initially discouraging but subsequent sequence alignment and clustering analysis strongly suggested that this putative site might indeed be a site of redox regulation in the serine–threonine phosphatase-1 subfamily[43]. If confirmed by experiment, this result will highlight the advantages of using structural descriptors to analyse multiple functional sites in proteins. It will also highlight the fact that human

Box 2. Knowing a protein's structure does not necessarily tell you its function

Because proteins can have similar folds but different functions[68,69], determining the structure of a protein may or may not tell you something about its function. The most well-studied example is the $(\alpha/\beta)_8$ barrel enzymes, of which triose-phosphate isomerase (TIM) is the archetypal representative. Members of this family have similar overall structures but different functions, including different active sites, substrate specificities and cofactor requirements[70,71].

Is this example common? Our own analysis of the 1997 SCOP database[68] shows that the five largest fold families are the ferredoxin-like, the (α/β) barrels, the knottins, the immunoglobulin-like and the flavodoxin-like fold families with 22, 18, 13, 9 and 9 subfamilies, respectively (Fig. i). In fact, 57 of the SCOP fold families consist of multiple superfamilies. These data only show the tip of the iceberg, because each superfamily is further composed of protein families and each individual family can have radically different functions. For example, the ferredoxin-like superfamily contains families identified as Fe–S ferredoxins, ribosomal proteins, DNA-binding proteins and phosphatases, among others.

After this article was submitted, a much-more-detailed analysis of the SCOP database was published[72]. This finds a broad function–structure correlation for some structural classes, but also finds a number of ubiquitous functions and structures that occur across a number of families. The article provides a useful analysis of the confidence with which structure and function can be correlated[72]. Knowing the protein structure by itself is insufficient to annotate a number of functional classes and is also insufficient for annotating the specific details of protein function.
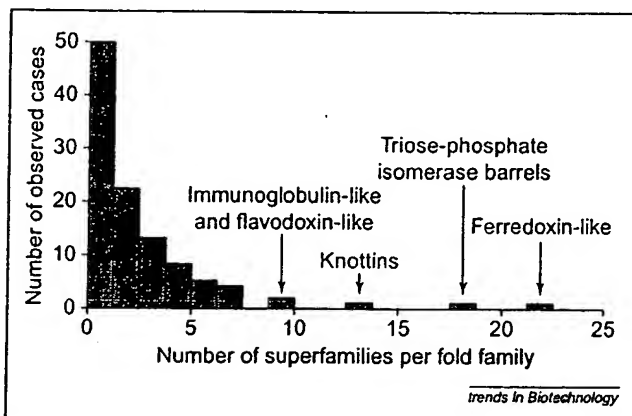

Figure i
Histogram of the numbers of superfamilies found in each SCOP fold family. These data clearly show that proteins with similar structures can have different functions and demonstrate the difficulty of assigning protein function based simply on the three-dimensional structure. The data were taken from the 1997 distribution of SCOP (http://scop.mrc-lmb.cam.ac.uk/scop). For a more-detailed analysis, see Ref. 72.

observation alone is no longer adequate for identifying all functional sites in known protein structures.

To date, the use of structure to identify function has largely focused on high-resolution structures and highly detailed descriptors of protein functional sites. However, the creation of inexact descriptors for functional sites opens the way to the application of these methods to inexact, predicted protein models. The question remains: how good does a model have to be in order to use FFFs to identify its active sites?

The state of the art in structure-prediction methods

For proteins whose sequence identity is above ~30%, one can use homology modeling to build the structure[44]. However, structure prediction is far more difficult for proteins that are not homologous to proteins with known structure. At present, there are two approaches for these sequences: ab initio folding[45-48] and threading[49-53].

In ab initio folding, one starts from a random conformation and then attempts to assemble the native structure. As this method does not rely on a library of pre-existing folds, it can be used to predict novel folds. The recent CASP3 protein-structure-prediction experiment (http://PredictionCenter.llnl.gov/CASP3) involved the blind prediction of the structure of proteins whose actual structure was about to be experimentally determined. These results indicate that considerable progress has been made[46,54]. For helical and α/β proteins with less than 110 residues, structures were often predicted whose backbone root-mean-square deviation (RMSD) from native ranged from 4–7 Å. Progress is being made with the β proteins, too, although they remain problematic. Because ab initio methods can identify novel folds, these methods could be used to help to select sequences likely to yield novel folds in experimental structural-genomics projects.

Another approach to tertiary-structure prediction is threading. Here, for the sequence of interest, one attempts to find the closest matching structure in a library of known folds[52,55]. Threading is applicable to proteins of up to 500 residues or so and is much faster than ab initio approaches. However, threading cannot be used to obtain novel folds.

Ab initio predicted models can be used for automatic protein-function prediction

The results of the recent CASP3 competition suggest that current modeling methods can often (but not always) create inexact protein models. Are these structures useful for identifying functional sites in proteins? Using the ab initio structure-prediction program MONSSTER, the tertiary structure of a glutaredoxin, 1ego, was predicted[56]. For the lowest-energy model, the overall backbone RMSD from the crystal structure was 5.7 Å.

To determine whether this inexact model could be used for function identification, the sets of correctly and incorrectly folded structures were screened with the FFF for disulfide-oxidoreductase activity[15]. The FFF uniquely identified the active site in the correctly folded structure but not in the incorrectly folded ones (Fig. 2). This is a proof-of-principle demonstration that inexact models produced by ab initio prediction of structure from sequence can be used for the subsequent prediction of biochemical function. Of course, improvements in the method have to be made before such predictions can be done on a routine basis.

Use of predicted structures from threading in protein-function prediction

At present, practical limitations preclude folding an entire genome of proteins using ab initio methods[57]. Threading is more appropriate for achieving the requisite high-throughput structure prediction. Thus, a standard threading algorithm[58] has been used to screen all

proteins in nine genomes for the disulfide-oxidoreductase active site described above.

First, sequences that aligned with the structures of known disulfide oxidoreductases were identified. Then, the structure was searched for matches to the active-site residues and geometry. For those sequences for which other homologs were available, a sequence-conservation profile was constructed[23]. If the putative active-site residues were not conserved in the sequence subfamily to which the protein belongs, that sequence was eliminated. Otherwise, the sequence is predicted to have the function.

Using this sequence-to-structure-to-function method, 99% of the proteins in the nine genomes that have known disulfide-oxidoreductase activity have been found. From 10% to 30% more functional predictions are made than by alternative sequence-based approaches; similar results are seen for the α/β hydrolases[23]. Surprisingly, in spite of the fact that threading algorithms have problems generating good sequence-to-structure alignments, active sites are often accurately aligned, even for very distant matches. This observation would agree with the above experimental results indicating that active sites are well conserved in protein structures.

Importantly, the false-positive rate when using structural information is much lower than that found using sequence-based approaches, as demonstrated by a detailed comparison of the FFF structural approach and the Blocks sequence-motif approach (N. Siew et al., unpublished). In this study, the sequences in eight genomes, including Bacillus subtilis, were analysed for disulfide-oxidoreductase function using the disulfide-oxidoreductase FFF, the thioredoxin Block 00194 and the glutaredoxin Block 00195. If we assume that those sequences identified by both the FFF and Blocks are 'true positives', we find 13 such sequences in the B. subtilis genome.

There is no experimental evidence validating all of these 'true positives' and so they are more accurately termed 'consensus positives'. In order to find these 13 'consensus positive' sequences, the FFF hits seven false positives. On the other hand, Blocks hits 23 false positives (Fig. 3). It was previously suggested that the use of a functional requirement adds information to threading and reduces the number of false positives[52]. These data, including the data shown in Fig. 3, validate this claim on a genome-wide basis.

Of course, as no genome has had the function of all of its proteins experimentally annotated, it is impossible to know how many other proteins with the specified biochemical function were not properly identified. This is a critical question for researchers attempting to predict protein function. Experimental confirmation will be needed to validate this or any other method fully. This points out the need for closely coupling computational function-prediction algorithms with experiments.

Weaknesses of using the sequence-to-structure-to-function method of function prediction

Based on studies to date, the identification of enzymatic activity requires a model in which the backbone RMSD from native near the active sites is about 4–5 Å. Predicted models are better at describing the geometry in the core of the molecule than in the loops and so
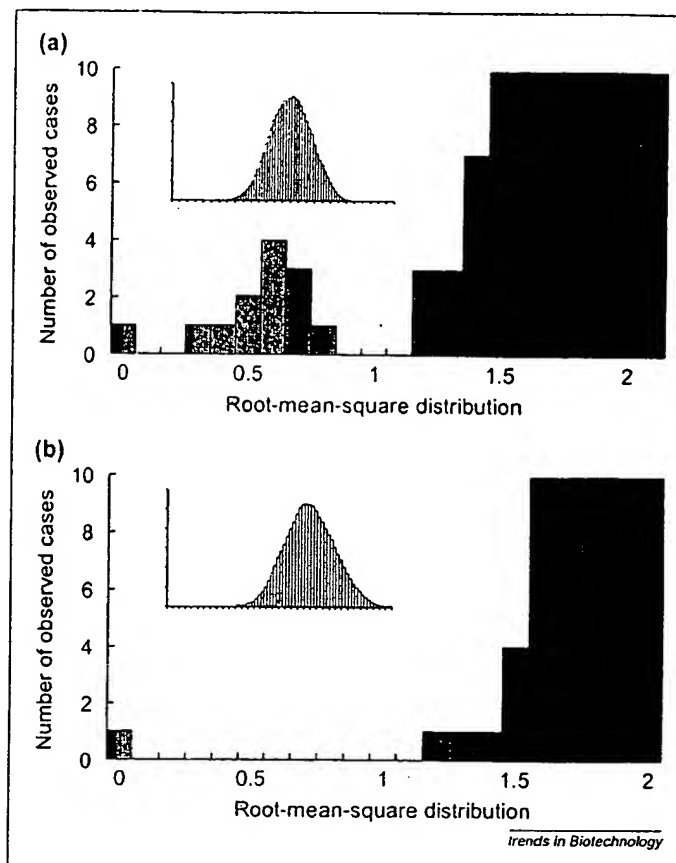
Figure 1

The distribution of root-mean-square distributions (RMSD) between the hydrolase catalytic triad and all other histidine-containing triads shows a bimodal distribution (a); by contrast, the RMSD between a randomly selected (non-catalytic) triad and all other histidine-containing triads has a unimodal distribution (b). The His–Ser–Asp catalytic triad in the protein-1 gpl (Rp2 lipase) (a) and a random histidine-containing triad from 4pga (glutaminase–asparaginase) (b) were structurally aligned to all His-containing triads in a database of 1037 proteins[23]. Actual α/β-hydrolase active sites (a) and the 4pga site (b) are indicated by blue bars; other histidine triads that are not active sites are indicated by red bars. None of the sites found by matching to the 4pga were hydrolase active sites. Inset graphs show the full distribution.

predicting the function of a protein whose active site is in loops may be a problem. Also, the method can currently only be applied to enzyme active sites; substrate- and ligand-binding sites have not been identified using the inexact models. Techniques that will further refine inexact protein models will be quite useful in taking the protein analysis to the next step.

Conclusions

Although sequence-based approaches to protein-function prediction have proved to be very useful, alternatives are needed to assign the biochemical function of the 30–50% of proteins whose function cannot be assigned by any current methods. One emerging approach involves the sequence-to-structure-to-function paradigm. Such structures might be provided by structural-genomics projects or by structure-prediction algorithms. Functional assignment is made by screening the resulting structure against a library of structural descriptors for known active sites or binding regions.
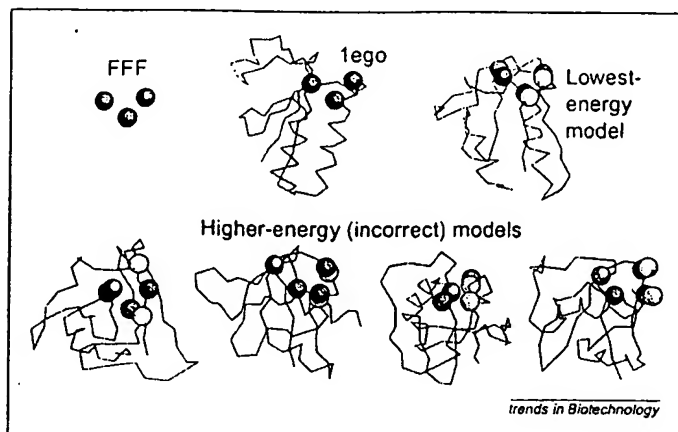
Figure 2

Application of the disulfide-oxidoreductase fuzzy functional form (FFF) to *ab initio* models of glutaredoxin created by the program MONSSTER shows that the FFF can distinguish between correctly folded and misfolded (or higher-energy) models. The FFF is shown as two orange balls (representing the cysteines) and a blue ball (representing the proline). The protein models are shown as magenta wire models with the active-site cysteines and proline shown as yellow and cyan balls, respectively. The FFF clearly distinguishes the correct active site in the crystal structure of the glutaredoxin 1ego and the correctly folded, lowest-energy model. The FFF does not match to the active sites of any of the higher energy, misfolded structures, four of which are shown here.
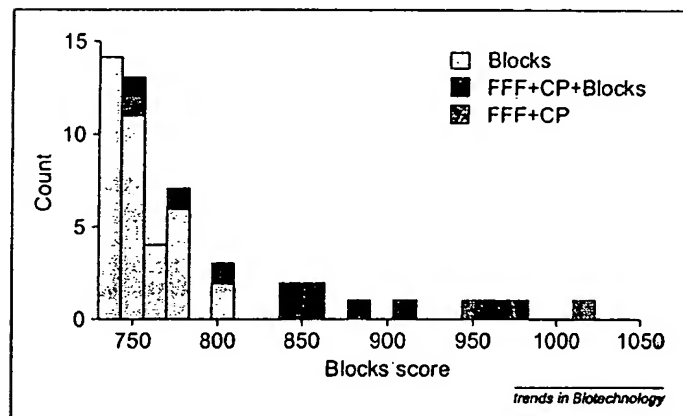
Figure 3

Analysis of the *Bacillus subtilis* genome using the thioredoxin Block 00194. The Blocks score (computed using the publicly available BLIMPS program) is plotted on the x axis and the number of sequences found in each scoring bin is plotted on the y axis. Those sequences identified as 'consensus positives' [identified by both the fuzzy functional form (FFF) and the Block] are shown as red bars. One additional sequence found by the FFF, which is likely to be a true positive, is shown as a blue bar. All other sequences, putative 'false positives', are shown as yellow bars. Using the Blocks score at which all 13 of the 'consensus positives' are found, 23 false positives are also found. In its analysis of the *B. subtilis* genome, the FFF identifies only seven false positives along with the same 13 'consensus positives' (data not shown).

Detailed descriptors will only work on the experimentally determined, high-quality structures. Ideally, however, the descriptors should work on both experimental structures and the cruder models provided by tertiary-structure-prediction algorithms.

The advantages of such an approach are that one need not establish an evolutionary relationship in order to assign function, that more than one function can be

assigned to a given protein [an issue of major importance, because proteins are multifunctional (Box 1)] and, ultimately, that having a structure can provide deeper insight into the biological mechanism of protein function and regulation. The disadvantages are that one needs to have the protein's structure before a function can be assigned and that the approach is limited to those functions associated with proteins with at least one solved structure, so that a functional-site descriptor can be constructed.

In this sense, structure-to-function assignment can be thought of as 'functional threading' – find the active-site match in a library of descriptors for known protein active sites. This is the first step in the long process of using structure to assign all levels of function, a goal that is made increasingly important with the emergence of structural genomics. Based on the progress to date, it is apparent that structure will play an important role in the post-genomic era of biology.

Acknowledgment

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EXHIBIT 6

1

Characteristics of the amino acids as components of a peptide hormone sequence

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In the living organism the polypeptide chains of proteins are used for the most diverse purposes: structural support and protection, catalysis of a wide range of chemical reactions, energy transduction, food storage, transport, and—among many others—regulation and co-ordination by information transfer (including humoral transmission by hormones). Molecules with the varied properties required for these multifarious functions are built up of a mere 20 units: the protein-constituent amino acids, connected primarily in a single structural mode (a linear sequence) but finally arranged in space in intricate ways to molecules of the required size, shape and properties. Although the peptide hormones fulfil such a highly specialised role, there is nothing to distinguish, *a priori*, their sequences from other polypeptide or protein sequences with different biological functions, or no function at all.

THE AMINO ACIDS

The 'proteinogenic' amino acids, though few in number, exhibit between them a remarkable range of chemical, physical and steric features. They are arranged in figure 1.1 in such a way that lines can be drawn to indicate their classification according to various properties. For instance, the sidechains may be hydrophilic (outside the hook-shaped line) or hydrophobic (within the hook); glycine, lying on the line, is taken as the reference amino acid for this purpose.

Again, some sidechains are chemically inert (those to the left of the broken line), while those to the right show varying kinds and degrees of chemical reactivity and may be capable of substitution, hydrogen bond or salt formation, oxidation, etc. Other classifications are shown by the use of frames. Some of the reactive sidechains are neutral, but others (shown by the lower right-hand frame) are charged either positively or negatively in the physio-

I. Rudinger
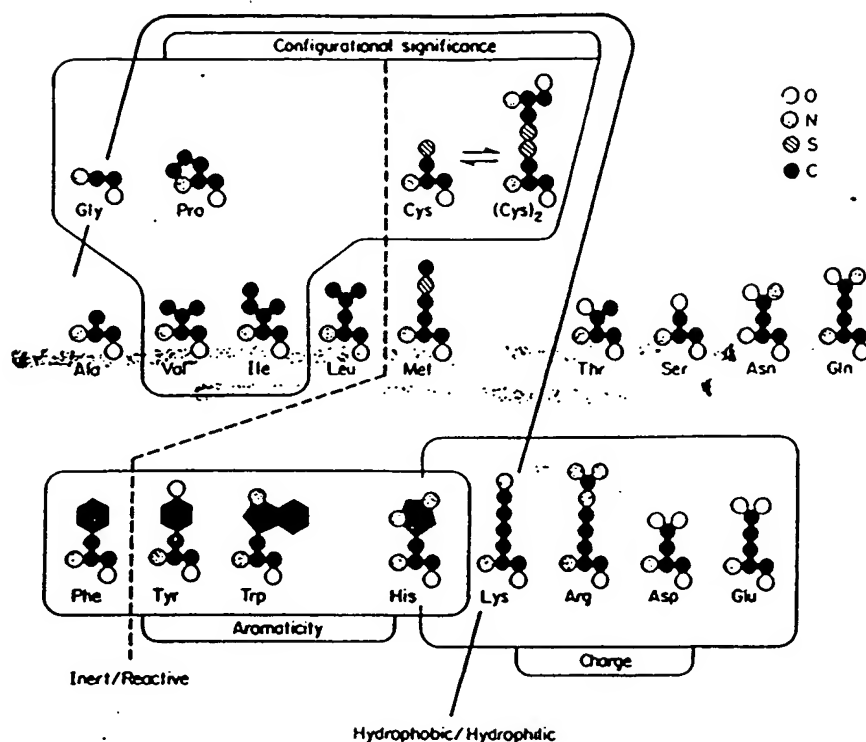
FIGURE 1.1 Schematic representation of the 20 amino acyl residues found in proteins, and their classification by certain properties; for details see text

confined to the *trans* geometr͔
cysteine sidechains provides ̣
linking and thereby stabili:
peptide chains.

It will be noted that most a
fying 'boxes'—in other words
features which may be utilis
taneously. As a result, it is im
residue in a sequence. A give
same 'significance' in differ
positions of the same sequen҄

For instance, isoleucine is f̣
and in position 5 of angioter.
may be replaced, without ap
β-branched amino acids pro
the steric requirements are n
the diastereomeric alloisoleu.
1972; Jorgensen and Weinka

Another illustration that s
provided by the molecule of
proline in position 3 by alani͏
assayed on the rabbit blood ͏
of proline are of no significan
substitution of the proline in
and in position 7 to 0.1 per c͏
sarcosine (*N*-methylglycine) ͏
these sites, the resulting ana
alanine derivatives (they re͏
activity of the parent compo͏

We may therefore conclu͏
sequence it is the *N*-alkylat

logical pH range. Histidine may or may not be protonated under physiological conditions. The two basic amino acids (lysine and arginine) differ in their equilibrium constants (arginine being the stronger base) and so do the two acidic amino acids (aspartic being a somewhat stronger acid than glutamic). Thus, between them, the charged amino acids encompass a wide range of pK values.

The lower left-hand frame encloses those sidechains which contain aromatic structures and exhibit corresponding special properties (π-electron interactions). Finally, the frame at the top draws attention to amino acids with special steric properties which affect the way in which the peptide chain can be arranged in three dimensions (its conformation or secondary structure). Glycine, with no obtruding sidechain, offers a particularly high degree of conformational freedom, whereas the bulky, β-branched sidechains of valine and isoleucine severely restrict the way in which the peptide chain may fold. A still greater degree of constraint is imposed by the rigid cyclic structure of proline. Proline has also the special property that the peptide bond in which its imino group participates may have either the *cis* or the *trans* conformation, whereas all other peptide bonds are normally

FIGURE 1.2 Sequences of

confined to the *trans* geometry. The formation of disulphide bridges between cysteine sidechains provides an even more positive way of covalently cross-linking and thereby stabilising the three-dimensional arrangement of peptide chains.

It will be noted that most amino acids occur in two or more of the classifying 'boxes'—in other words, each generally has several different structural features which may be utilised in protein building alternatively or simultaneously. As a result, it is impossible to attach a unique significance to any residue in a sequence. A given amino acid will not by any means have the same 'significance' in different peptide sequences, or even in different positions of the same sequence.

For instance, isoleucine is found both in position 3 of oxytocin (figure 1.2a) and in position 5 of angiotensin II (figure 1.2b). Whereas in angiotensin it may be replaced, without appreciable loss of biological activity, by other β-branched amino acids provided they are equally lipophilic, in oxytocin the steric requirements are much more stringent and even replacement by the diastereomeric alloisoleucine causes a drastic fall in activity (Rudinger, 1972; Jorgensen and Weinkam, 1973).

Another illustration that sidechain 'significance' depends on 'context' is provided by the molecule of bradykinin (figure 1. 2c). Replacement of the proline in position 3 by alanine does not affect the potency of the peptide as assayed on the rabbit blood pressure; obviously, the special steric properties of proline are of no significance in this position. On the other hand, the same substitution of the proline in position 2 reduces the activity to 0.5 per cent, and in position 7 to 0.1 per cent (Schröder and Hempel, 1964). Moreover, if sarcosine (*N*-methylglycine) rather than alanine is used to replace proline at these sites, the resulting analogues are considerably more active than the alanine derivatives (they retain, respectively, 50 and 30 per cent of the activity of the parent compound; Yanaihara *et al.*, 1966).

We may therefore conclude that in positions 2 and 7 of the bradykinin sequence it is the *N*-alkylation of the nitrogen in the peptide backbone
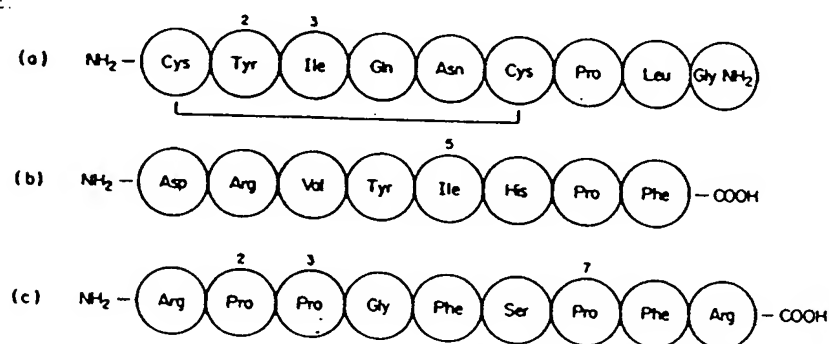
FIGURE 1.2 Sequences of oxytocin (a), angiotensin II (b) and bradykinin (c)

which is the important feature of the proline structure rather than, for example, the presence of its ring or its lipophilic properties.

SEQUENCE AND CONFORMATION

The humoral mechanism of information transfer requires that the effect of a hormone on its receptor be both sensitive and specific. These requirements can be met only by hormone–receptor binding based on multiple inter-actions of complementary sites: it is a *pattern* on the hormone molecule which is 'recognised' by the receptor in the binding process. Generally, parts of several amino acids of the sequence will participate in forming this pattern. In peptide molecules which are short or conformationally very flexible, or both, the pattern will often involve the sidechains of amino acids which are close together in the primary peptide sequence ('continuate' or 'synchnologic' read-out (Schwyzer, 1972), analogous to 'sequential' determinants in antigens (Sela, 1969)).

However, in peptides whose conformation is stabilised either by a sufficient number of intramolecular, non-covalent sidechain interactions or by disulphide bonds, or both, the critical topochemical pattern may be made up of groups widely separated in the linear sequence (Hofmann and Katsoy-annis, 1963; Schwyzer, 1963; Rudinger and Jošt, 1964) ('discontinuate' or 'rhegnylogic' read-out, analogous to 'conformational' determinants).

Whereas in the first case the often-cited analogy to a 'message' written in linear, alphabetic script is valid, a topochemical arrangement of the second type is better likened to Chinese writing: it is the *pattern* of the character which conveys the meaning (figure 1.3) and not the (prescribed) order in which the brush strokes are made.

In either case it should be noted that the conformation of the hormone molecule in its interaction with the receptor need not be identical with its conformation in solution (cf. Rudinger and Jošt, 1964; Rudinger, 1972),
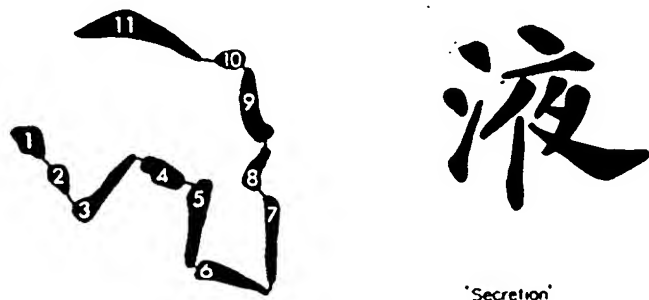
'Secretion'

FIGURE 1.3 Eleven brush-strokes in the order in which they are made but in random pattern (left), and in the pattern in which they make up the Chinese character for 'secretion'

although major, energetically disfavou admittedly unlikely.

SIGNIFIC

In a given molecule some amino acid: 'significance' to their inclusion in the in recognition by, and binding to, the i existence of this pattern is dependen intramolecular interactions, as discuss acids or sequences contributing to this less 'significant' for the biological ac generally, sequences contributing to tl affect its transport and distribution, m non-receptor sites, etc., may signific: In defining the relation between sequer into account all these contributions, ai in receptor binding*.

Two separate events may be conc receptor interaction: binding (recogn the signal which eventually leads to tl models proposed to account for hon generation is actually identical with the of the 'allosteric' model (Monod, Cha common the assumption that bindin: activity of a second, topologically di bably by inducing a conformational c tion' type; Rudinger, Pliška and Kr ration are two distinct molecular even

The properties of a series of oxytc residue (position 2) illustrate a po problem and favour a 'participatic figure 1.4, replacement of the hydr substituents leads, in a graded manne the appearance of inhibitor properti from pharmacological parameters (r the whole series of analogues, sug; around position 2 is involved in st to binding (see Rudinger *et al.*, 1972 substitution *ortho* to the tyrosine hy methyl—also gives rise to inhibi

*Obviously, these various functions need not and distinct sequences, but particular amin participate in several of them.

although major, energetically disfavoured structural rearrangements are admittedly unlikely.

SIGNIFICANCE

In a given molecule some amino acids or sequences obviously owe their 'significance' to their inclusion in the pattern which is directly involved in recognition by, and binding to, the receptor. However, the fact that the existence of this pattern is dependent on a conformation stabilised by intramolecular interactions, as discussed above, implies that other amino acids or sequences contributing to this conformational stability will be no less 'significant' for the biological activity of the molecule. Even more generally, sequences contributing to the e propei .ies of the peptide which affect its transport and distribution, metabolic transformations, binding to non-receptor sites, etc., may significantly modify its biological activity. In defining the relation between sequence and activity it is necessary to take into account all these contributions, and not merely those directly involved in receptor binding*.

Two separate events may be conceptually distinguished in hormone-receptor interaction: binding (recognition) and stimulation (initiation of the signal which eventually leads to the observed response). In some of the models proposed to account for hormone action, the process of stimulus generation is actually identical with the process of binding. These are variants of the 'allosteric' model (Monod, Changeux and Jacob, 1963), which have in common the assumption that binding of hormone at one site modifies the activity of a second, topologically distinct site on the same molecule, probably by inducing a conformational change. In other models (the 'participation' type; Rudinger, Pliška and Krejčí, 1972) binding and stimulus generation are two distinct molecular events.

The properties of a series of oxytocin analogues modified at the tyrosine residue (position 2) illustrate a possible experimental approach to this problem and favour a 'participation' model for oxytocin. As shown in figure 1.4, replacement of the hydroxyl group of this tyrosine by various substituents leads, in a graded manner, to loss of oxytocin-like activity and to the appearance of inhibitor properties. Yet the binding affinity, determined from pharmacological parameters (pD_2 and pA_2), is practically the same for the whole series of analogues, suggesting that the region of the molecule around position 2 is involved in stimulus generation but contributes little to binding (see Rudinger *et al.*, 1972). Moreover, we have recently found that substitution *ortho* to the tyrosine hydroxyl group—for example, by iodine or methyl—also gives rise to inhibitors: evidently the hydroxyl group is

*Obviously, these various functions need not and, in general, will not be attributable to separate and distinct sequences, but particular amino acids, sequences or topochemical regions may participate in several of them.
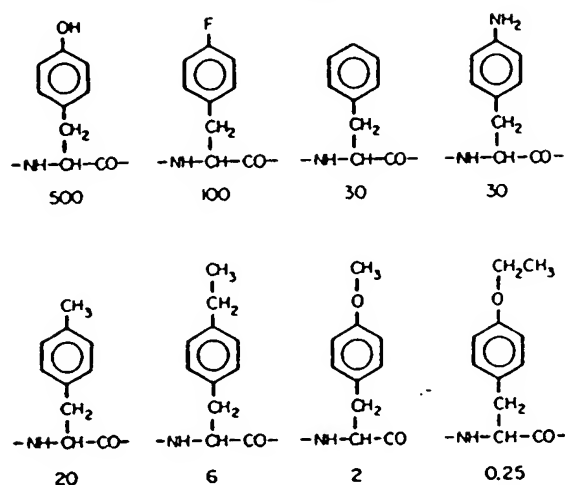
FIGURE 1.4 Replacement of the tyrosine sidechain (top left) by other, modified sidechains in analogues of oxytocin. The figures give the uterotonic activity in IU/mg under standard assay conditions (see Rudinger *et al.,* 1972; Marbach and Rudinger, 1974a)

displaced from its functionally required alignment by the substituents, but once more the binding affinity, as measured by the inhibitory constants, remains high (Marbach and Rudinger, 1974b).

CONCLUSIONS

The significance of particular amino acids and sequences for different aspects of biological activity cannot be predicted *a priori* but must be determined from case to case by painstaking experimental study. The careful design of synthetic analogues, and their evaluation in biological systems which permit separate analysis of the various phases of hormone action, is still the best way of obtaining such information.

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EXHIBIT 7

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
    Chada et al.

Serial No.: 10/017,472

Filed: December 7, 2001

For: METHODS OF TREATMENT
    INVOLVING HUMAN MDA-7

Group Art Unit: 1632

Examiner: Li, Qian J.

Atty. Dkt. No.: INGN:097US

DECLARATION OF SUNIL CHADA, Ph.D

I, Sunil Chada, declare:

1.   I am the Director of Research and Development at Introgen Therapeutics. I have been working in the field of gene therapy and cancer biology for at least 15 years. My *curriculum vitae* is attached as Exhibit 1.

2.   I am also one of the inventors named on the application identified above, which concerns the melanoma differentiation associated gene (mda-7) and its encoded protein, MDA-7.

3.   The *mda-7* gene was first identified in human melanoma cell lines as a possible tumor suppressor. Jiang *et al.*, *Oncogene* 11:2477-86 (1995). Subsequent studies confirmed that elevated levels of MDA-7 suppressed cancer cell growth *in vitro* and selectively induced apoptosis in human breast cancer cells and inhibited tumorigenicity in nude

mice. Jiang *et al., Proc. Nat'l. Acad. Sci.* 93:9160-65 (1996); Su *et al., Proc. Nat'l Acad. Sci.* 95:14400-05 (1998).

4. I understand that the present application contains claims directed to methods of inhibiting angiogenesis involving administering a nucleic acid expressing the human MDA-7 polypeptide, which have been rejected as lacking enablement.

5. As described in this application, the first 48 amino acids of the full-length sequence may be cleaved to yield a secreted form of the protein. I have done scientific research on the tumor suppressor gene mda-7 and the MDA-7 protein, both the full-length and truncated versions.

6. In one study concerning the MDA-7 protein, human melanoma cell lines MeWo and WM35 were treated with increasing concentrations of an MDA-7 protein lacking the first 48 amino acids of the full-length sequence. The cell lines were analyzed in triplicate at 12, 24, 48, 72, and 96 hours after treatment using a trypan blue exclusion assay. This truncated MDA-7 protein induced cell killing in melanoma cells (Exhibit 2), but did not induce killing in lung cancer cells.

7. In another study, different forms of the MDA-7 protein were evaluated in PC3 human prostate cancer cells and H1299 human non-small cell lung carcinoma cells. The different forms (Exhibit 3) included: a full-length MDA-7, an MDA-7 protein lacking its own secretion signal (cytoplasmic version. lacking first 48 amino acids), an MDA-7 targeted to the nucleus (nuclear version), and an MDA-7 lacking its own secretion signal but containing a signal targeting it to the endoplasmic reticulum (ER version). Cells transfected with either the full-length or ER version of MDA-7 showed growth suppression (Exhibit 4). Furthermore, there were higher levels of apoptosis observed in

cells transfected with the full-length or ER versions, as compared to the cytoplasmic or nuclear versions of MDA-7.

8.   Thus, as discussed in paragraph 6, the truncated version of MDA-7 does indeed induce apoptosis as set forth in the specification of this application. Furthermore, as discussed in paragraph 7, a truncated MDA-7 with a heterologous signal sequence suppresses growth and induces apoptosis.

9.   Moreover, while the specification provides data regarding an Ad-mda7 construct to express MDA-7 in a eukaryotic cell, another study involved formulating a plasmid with an MDA-7 encoding nucleic acid in a liposome composition. The human mda-7 cDNA was placed under the control of the CMV promoter in a plasmid, which was formulated in a DOTAP:cholesterol complex. Nude mice were injected with human non-small cell lung carcinoma cells (A549 cell line) to produce tumors. Tumors were then treated intratumorally with the DOTAP:Chol-*mda-7* complex (50 µg/dose), resulting in the inhibition of tumor growth as compared to tumors in control animals. Similarly, tumors in nude mice from implantation of fibrosarcoma cells (UV223M cells) (syngeneic tumor model) were also inhibited by intratumoral administration of the DOTAP:Chol-*mda-7* complex. Moreover, when the tumor tissue from these animals were evaluated for CD31, they exhibited reduced levels of staining, which is indicative of reduced vascularization.

10.   I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such

willful false statements may jeopardize the validity of this application or any patent issued thereon.

_01 - 29 - 04_
Date

Sunil Chada, Ph.D.

4

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. DO NOT EXCEED FOUR PAGES.

NAME	POSITION TITLE
SUNIL CHADA	DIRECTOR OF RESEARCH AND DEVELOPMENT

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Kings College, University of London London, England	B.Sc. (Honors)	1982	Cell & Molecular Biology
University of California at Los Angeles Los Angeles, CA	M.Sc.	1985	Molecular Biology
University of Massachussetts Medical School Worcester, MA	Ph.D.	1988	Molecular Genetics

A.

B. Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

PROFESSIONAL EXPERIENCE

1985-1988	Research Associate, Univ. of Massachusetts Medical School, Worcester MA
1988-1991	Research Scientist I, Dept. of Molecular Virology, Viagene Inc., San Diego CA
1991-1993	Research Scientist II, Dept. of Immunobiology, Viagene Inc., San Diego CA
1993-1995	Senior Scientist, Dept. of Immunobiology, Viagene Inc., San Diego CA
1995-1997	Staff Scientist, Chiron Technologies Inc., San Diego CA
1997-pres	Director of Research and Development, Introgen Therapeutics, Houston TX
2002-pres	Adjunct Faculty, Dept. of Bioimmunotherapy, Division of Cancer Medicine, MD Anderson Cancer Center

Committee Memberships

National Cancer Institute – SBIR/ STTR SRG Reviewer (standing member)
National Cancer Institute – Cancer Chemoprevention (Ad hoc member)
National Cancer Institute – RAID Committee member
Rice University – Advisory Board for NIH and NSF Biotechnology Training Programs
Alliance for Cancer Gene Therapy - Reviewer

C. Selected peer-reviewed publications (from a total of 68).

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C. Research Support.
GRANTS AWARDED (from a total of 9)

1. Chada S. "Novel Gene Therapeutic for the Treatment of Lung Cancer" SBIR Grant 1R43CA86587-01 (Funded 03/00). Role: PI. Goals: to evaluate Ad-mda7 as a potential therapeutic for NSCLC

2. Meyn R "Tumor cell radiosensitization by gene drugs" STTR Grant. (Funded 08/00). Role: co-PI. Goal: to evaluate radiosensitization by Ad-p16 and Ad-mda7

3. Grimm EA "Novel gene therapy for Melanoma" STTR grant (Funded 06/01). Role: co-PI. Goals: to evaluate Ad-mda7 as a potential therapeutic for melanoma.

4. Chada S "Combination treatment for breast cancer using Ad-mda7 plus Herceptin". SBIR grant (Funded 07/02). Role: PI. Goals: To evaluate synergy between Ad-mda7 and Herceptin in breast cancer

5. Grimm EA "Phase II clinical trial for Melanoma using INGN 241 (Ad-mda7)" STTR grant (Funded 09/03). Role: co-PI

PATENTS and APPLICATIONS 8 issued patents; 17 applications pending

MDA-7 induces cell killing in Melanoma cells

% Killing (Trypan Blue)

WM35

12 Hours
24 Hours
48 Hours
72 Hours
96 Hours

☐ 0 ng
■ 5 ng
■ 10 ng
■ 20 ng

Secretion signal   "processed" protein   Myc tag   Predicted Size

(1) FL   25 kD

(2) Cyto   21 kD

(3) Nuc   3X NLS   31 kD

ER signal peptide   ER retention signal

(4) ER   22 kD

A Single Intramuscular Injection of Recombinant Plasmid DNA Induces Protective Immunity and Prevents Japanese Encephalitis in Mice

GWONG-JEN J. CHANG,* ANN R. HUNT, AND BRENT DAVIS

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Plasmid vectors containing Japanese encephalitis virus (JEV) premembrane (prM) and envelope (E) genes were constructed that expressed prM and E proteins under the control of a cytomegalovirus immediate-early gene promoter. COS-1 cells transformed with this plasmid vector (JE-4B clone) secreted JEV-specific extracellular particles (EPs) into the culture media. Groups of outbred ICR mice were given one or two doses of recombinant plasmid DNA or two doses of the commercial vaccine JEVAX. All mice that received one or two doses of DNA vaccine maintained JEV-specific antibodies 18 months after initial immunization. JEVAX induced 100% seroconversion in 3-week-old mice; however, none of the 3-day-old mice had enzyme-linked immunosorbent assay titers higher than 1:400. Female mice immunized with this DNA vaccine developed plaque reduction neutralization antibody titers of between 1:20 and 1:160 and provided 45 to 100% passive protection to their progeny following intraperitoneal challenge with 5,000 PFU of virulent JEV strain SA14. Seven-week-old adult mice that had received a single dose of JEV DNA vaccine when 3 days of age were completely protected from a 50,000-PFU JEV intraperitoneal challenge. These results demonstrate that a recombinant plasmid DNA which produced JEV EPs in vitro is an effective vaccine.

Japanese encephalitis (JE) is a mosquito-borne viral disease of major public health importance in Asia. More than 35,000 cases and 10,000 deaths are reported annually (52). *Japanese encephalitis virus* (JEV) is a member of the genus *Flavivirus* in the family *Flaviviridae*. More than 70 species in the *Flavivirus* genus have been genetically and serologically classified (29). Other important human pathogenic flaviviruses include yellow fever, dengue type 1 to 4 (DEN1 to DEN4), tick-borne encephalitis (TBE), and St. Louis encephalitis (SLE) viruses. Vaccination has been an effective mechanism for prevention of flavivirus infection in humans and domestic animals. Three JEV vaccines are in widespread production and use (52). These are inactivated virus from infected mouse brain, inactivated virus from primary hamster kidney cells, and a live attenuated SA14-14-2 vaccine. Only inactivated JEV vaccine, JEVAX, produced in mouse brain is distributed commercially and available internationally (52). Inactivated, mouse brain-derived whole virus vaccine is costly to prepare and carries the risk of allergic reaction to murine encephalitogenic basic proteins or gelatin stabilizer (45; M. M. Andersen, and T. Ronne, Letter, Lancet 337:1044, 1991). Since 1989, an unusual number of systemic reactions characterized by generalized urticaria and/or angioedema following JEVAX immunization have been reported from Australia, Canada, and Denmark (36). A major problem associated with use of the inactivated mouse brain vaccine is the failure to stimulate long-term immunity (39). Multiple immunization is recommended to provide adequate protection (28, 39). The attenuated JEV vaccine, SA14-14-2, is undergoing clinical trials (31). However, because of regulatory issues this vaccine has not found wide acceptance outside the People's Republic of China (11).

Several experimental recombinant virus, attenuated virus, and subunit JEV vaccines have been reported. Recombinant baculovirus vector that contained the JEV envelope (E) protein gene has been used to infect insect cells and produce E protein that has been studied as a biosynthetic immunogen (33). Recombinant vaccinia viruses expressing the JEV genes extending from premembrane (prM) to NS2B proteins have been the most promising candidate vaccines. These candidate vaccines produced extracellular virus-like particles (EPs) in infected cell culture that induced high titers of neutralizing and hemagglutination-inhibiting antibodies and protective immunity in mice (19–21, 47, 54). Recombinant vaccinia viruses expressing the same JEV genes based on the attenuated vaccinia virus strain, NYVAC-JEV, or canarypox, ALVAC-JEV, were tested in phase I human trials (18). In this trial, only 1 in 10 ALVAC-JEV recipients developed detectable viral neutralizing antibody, and vaccinia virus-preimmune recipients had a significantly lower humoral immune response.

Inoculation of animals with purified plasmid vectors (DNA) by the intramuscular (i.m.) or intradermal route leads to expression of the recombinant vector-encoded protein in transfected cells, resulting in stimulation of a protein-specific immune response. Plasmid DNA vaccines provide an alternative to attenuated, inactivated, or virus-vectored subunit vaccines. Flavivirus DNA vaccines for Murray Valley encephalitis, DEN2, JE, SLE, and TBE (Central European encephalitis and Russian spring summer encephalitis) viruses have been developed and tested in the mouse model (4, 17, 24, 30, 38, 49). All of these plasmid DNA constructs contained similar transcriptional regulatory elements and a flavivirus gene cassette. Vaccination of mice with these plasmid DNA vaccines induced a virus-specific antibody response, as detected by enzyme-linked immunosorbent assay (ELISA). However, production of neutralizing antibody leading to 100% protection of vaccinated animals from virus challenge was observed only after multiple immunizations or delivery of DNA to the epidermis by particle

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FIG. 1. Map of the JEV genomic structure (top) and the DNA sequence of oligonucleotides used in RT-PCR to construct the transcription unit for the expression of prM-E protein coding regions (bottom). Potential transmembrane helices of viral polyprotein are indicated by blackened areas.

bombardment (4, 24, 49). In this study, we constructed a JEV prM and E gene cassette that incorporates an extended signal peptide sequence at the NH$_2$ terminus of the prM gene and Kozak's sequence, an optimal translation enhancing element surrounding the AUG site. JEV protein expression was characterized using six different recombinant vectors containing the same insert. The humoral immune response and protection from virulent JEV challenge following immunization with the recombinant plasmid DNAs were compared to findings for the human vaccine, JEVAX, licensed by the U.S. Food and Drug Administration, in outbred ICR mice.

MATERIALS AND METHODS

Cell culture and virus strains. COS-1, COS-7, and SV-T2 cells (1650-CRL, 1651-CRL, and 163.1-CCL; American Type Culture Collection) were grown at 37°C in Dulbecco's modified Eagle medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 7.5% NaHCO$_3$, (30 ml/liter), penicillin (100 U/ml), streptomycin (100 μg/ml). COS-1 and COS-7 cells were derived from simian virus 40 (SV40) transformed CV1 cells which have an African green monkey kidney cell origin. SV-T2 cells were derived from SV40-transformed mouse fibroblasts. Vero cells were grown under the same conditions except that 5% fetal calf serum without nonessential amino acid was used. C6/36 cells (13) were grown at 28°C in the same medium used for the COS-1 cells. The SA14 strain of JEV, propagated by intracranial inoculation into suckling mouse brain, was used for animal challenges and plaque reduction neutralization tests (PRNT). The SA14 virus used in ELISA and Western blot experiments was propagated in C6/36 cells and purified by ultracentrifugation on 30% glycerol-45% potassium tartrate gradients (37).

Construction of plasmids expressing JEV prM and E gene proteins. Genomic RNA was extracted from 150 μl of SA14 mouse brain JEV by using a QIAamp viral RNA kit (Qiagen, Santa Clarita, Calif.). RNA was adsorbed on a silica membrane, eluted in 80 μl of diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.)-treated water, and used as a template for amplification of JEV prM and E genes. Primer sequences were obtained from the published data (35). A single cDNA fragment containing genomic nucleotides (nt) 389 to 2478 was amplified by reverse transcriptase-mediated PCR (RT-PCR). Restriction enzyme sites for KpnI and XbaI and Kozak's sequence for an optimal translation initiation (25, 26) were engineered at the 5' terminus of the cDNA by amplimer 14DV389. An in-frame translation termination codon, followed by a NotI restriction site, was introduced at the 3' terminus of the cDNA by amplimer

c14DV2453 (Fig. 1). A single-tube RT-PCR was performed using a Titan RT-PCR Kit (Roche Molecular Biochemical, Indianapolis, Ind.). The RT-PCR product was purified using a QIAquick PCR purification kit (Qiagen), and the DNA was eluted with 50 μl of 1 mM Tris-HCl (pH 7.5).

All vector constructions and analyses were carried out using standard techniques (46). RT-PCR-amplified cDNA was digested with enzymes KpnI and NotI and inserted into the KpnI-NotI site of eukaryotic expression plasmid vector pCDNA3 (Invitrogen, Carlsbad, Calif.). Electroporation-competent Escherichia coli XL1-Blue cells (Stratagene, La Jolla, Calif.) were transformed by electroporation (Gene Pulser; Bio-Rad Laboratories, Hercules, Calif.) and plated on Luria broth (LB) agar plates that contained carbenicillin (100 μg/ml; Sigma). Clones were picked and inoculated into 3 ml of LB containing carbenicillin (100 μg/ml). Plasmid DNA was extracted from a 14-h LB culture by using a QIAprep Spin Miniprep kit (Qiagen). Automated DNA sequencing was performed as recommended on an ABI Prism 377 DNA sequencer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.). Both strands of the cDNA were sequenced and compared to the published SA14 virus sequence (35).

The pCDNA3 fragment from nt 1289 to nt 3455, which contained the fl-encoded eukaryotic origin of replication (ori), SV40 ori, neomycin coding region, and SV40 poly(A) elements, was deleted by PvuII digestion and then self-ligated to generate plasmid pCBamp. The pCIBamp vector, which contained a chimeric intron insertion at the NcoI-KpnI site of the pCB vector, was constructed by excising the intron sequence from pCI (Promega, Madison, Wis.) by digestion with NcoI and KpnI. The resulting 566-bp fragment was cloned into NcoI-KpnI-digested pCBamp to replace its 289-bp fragment. Figure 2 shows a schematic drawing of plasmids pCDNA3, pCBamp, and pCIBamp.

The DNA fragment containing the JEV coding region in the recombinant plasmid pCDJB2-7, derived from the pCDNA3 vector, was excised by NotI and KpnI or XbaI digestion and cloned into the KpnI-NotI sites of pCB, pCIB, pCEP4 (Invitrogen), and pREP4 (Invitrogen) and into the SpeI-NotI site of the pRc/RSV (Invitrogen) expression vector to create pCBJB1-14, pCIBJES14, pCBJB, pREJB, and pRCJB, respectively. Both strands of the cDNA from each plasmid vector were sequenced, and recombinant clones with a correct nucleotide sequence were identified. Plasmid DNA for in vitro transformation or mouse immunization was purified by anion-exchange chromatography using an Endo-Free Plasmid Maxi kit (Qiagen).

IFA. Expression of JEV-specific gene products by the various recombinant expression plasmids was evaluated by indirect immunofluorescence antibody assay (IFA) in the transient expression system using COS-1, COS-7, and SV-T2 cells. For transformation, cells were grown to 75% confluence in 150-cm^2 culture flasks, trypsinized, and resuspended in 4°C phosphate-buffered saline (PBS) to a final density of 1 × 10^7 to 2 × 10^7 cells/ml. Five hundred microliters of cell suspension was then electroporated with 10 μg of plasmid DNA, using a Bio-Rad Gene Pulser II set at 250 V and 960 μF. Cells were diluted with 25 ml of fresh medium after electroporation and seeded into one 75-cm^2 flask. Forty-eight hours after transformation, the medium was removed, and the cells were

TATA box   KpnI   BamHI   EcoRI   EcoRV   NotI

TATATAA__(92nt)__GGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCTGCAGATATCCATCACACTGGCGGCCGC_BHG-poly(A)

FIG. 2. Schematic representations of plasmid vectors pCDNA3, pCBamp, and pCIBamp. These plasmids include the CMV promoter/enhancer element, BGH poly(A) signal and transcription termination sequence [BHGp(A)], ampicillin resistance gene (Amp), and ColE1 ori for selection and maintenance in *E. coli*. The fl ori for single-stranded rescue in *E. coli* cells, SV40 ori, neomycin coding region, and SV40 poly(A) [SV40 p(A)] sequences were deleted from pCDNA3 to generate pCBamp. An intron sequence was inserted in the *NcoI-KpnI* site of pCBamp to generate pCIBamp. The multiple cloning site for the insertion of JEV genes, located between the TATA box of the CMV promoter/enhancer and BHG poly(A) site, is shown.

trysinized and resuspended in 5 ml of PBS with 3% normal goat serum. Ten-microliter aliquots of the cell suspension were then spotted onto slides, air dried, and fixed with acetone at 4°C for 10 min. Immunofluorescent mapping of the E protein-specific epitopes was performed using a panel of murine monoclonal antibodies (MAbs) (15, 42, 55) and JEV-specific hyperimmune mouse ascitic fluid (HIAF). All antibodies were tested at 1:400 dilution in PBS.

Selection of an in vitro-transformed stable cell line constitutively expressing JEV-specific gene products. COS-1 cells transformed with 10 μg of pCDJE2-7 DNA by electroporation were incubated in nonselective culture medium for 24 h and then treated with neomycin (G418; 0.5 mg/ml; Sigma). G418-resistant colonies, which became visible after 2 to 3 weeks, were cloned by limited dilution in G418-containing medium. Expression of the JEV proteins was determined by IFA using JEV HIAF. One IFA-positive (JB-4B) and one IFA-negative (JB-5A) clone were selected for further analysis and maintained in medium containing 200 μg of G418 per ml. These stably transformed cells secreted antigen in the form of EPs (A. Hunt and G. J. Chang, unpublished data).

Antigen capture ELISA for detection of E protein secreted into culture fluid. The antigen capture ELISA, a modification of the procedure described by Guirakhoo et al. (8), was used to detect E protein from transiently transformed cells or JB-4B culture fluid. Flavivirus group-reactive MAb 4G2 was used to capture the JEV antigens (7). The 4G2-captured antigen was detected using horseradish peroxidase-conjugated MAb 6B6C-1 by incubation for 1 h at 37°C. Enzyme activity on the solid phase was detected with 3,3′,5,5′-tetramethylbenzidine ELISA substrate (Life Technologies, Grand Island, N.Y.); the reaction was stopped with the addition of 2 M H₂SO₄, and the optical density was measured at 450 nm.

Mouse experiments. Three-day-old mixed-sex or 3-week-old female ICR outbred mice were vaccinated i.m. with 50 or 100 μg of plasmid DNA at a concentration of 1 μg/μl in PBS or subcutaneously (s.c.) with 1/10 or 1/5 of the adult human dose of JEVAX (manufactured by the Research Foundation for Microbial Disease of Osaka University and distributed by Connaught Laboratories, Swiftwater, Pa.). The chloramphenicol acetyltransferase (CAT) protein expression plasmid pCDNA3/CAT (Invitrogen) was used as the vaccination control. Selected groups of mice were boosted 3 weeks later with an additional dose of plasmid vaccine or JEVAX. Mice were bled from the retro-orbital sinus; serum samples were evaluated for JEV antibody by ELISA and Western blotting using purified JEV and by PRNT.

Mice vaccinated at 3 days of age were challenged intraperitoneally (i.p.) 7 weeks postvaccination with JEV strain SA14 (50,000 PFU/100 μl) and observed for 3 weeks. To evaluate passive protection by maternal antibody, pups were obtained from mating of nonimmunized males with immunized females 9 weeks following their vaccination with plasmid DNA at 3 weeks of age. Pups were challenged by the i.p. route 3 to 15 days after birth with SA14 virus (5,000 PFU/100 μl) and observed daily for 3 weeks. Postchallenge serum was collected from survivors and tested for reactivity with JEV antigens by ELISA and Western blotting.

Serological tests. Postvaccination and postchallenge serum samples were tested for the ability to bind to purified JEV by ELISA, neutralize JEV infectivity by PRNT, or recognize JEV proteins by Western blotting (12, 41, 48). The PRNT assay was performed by incubating ~200 PFU of SA14 virus in 100 μl of Dulbecco's modified Eagle medium containing 5% bovine serum albumin and 20 mM HEPES buffer (pH 8.0) with serial twofold dilutions of serum specimens, started at 1:10, in 100 μl of the same buffer in 96-well trays at 4°C overnight. Serum specimens were heat inactivated at 56°C for 30 min before use. Duplicate 100-μl aliquots were assayed for infective virus by plaque formation on Vero cell monolayers. The percent plaque reduction was calculated relative to virus controls without serum. Titers were expressed as the reciprocal of serum dilutions yielding a 90% reduction in plaque number (PRNT₉₀).

RESULTS

Effect of the promoter and poly(A) signal on the efficiency of JEV prM and E protein expression. Four eukaryotic cell expression plasmids that contained the JEV coding region extending from genomic nt 390 to nt 2478 were constructed. This region of the genome encoded the prM and E genes. The Kozak sequence for the eukaryotic translation initiation site (underlined) of −9 to +4, GCCGCCGCCATGG, at the 5′ terminus (2, 25, 26, 27) and the in-frame translation terminat ion sequence at the 3′ terminus of cDNA were incorporated directly into cDNA by RT-PCR using viral RNA as a template. Transcription of the JEV genes in plasmid pCDJE2-7 was controlled by the human cytomegalovirus (CMV) early IA gene promoter/enhancer. The resulting mRNA is terminated and stabilized by a bovine growth hormone (BGH) transcript ion terminator and a poly(A) signal, respectively. The transcriptional control elements in pREJE were replaced by the Rous sarcoma virus (RSV) long terminal repeat promoter and SV40 poly(A). The pCEJE and pRCJE plasmids contain CMV plus SV40 poly(A) and RSV plus BGH poly(A), respectively (Table 1).

To determine the influence of the promoter and poly(A) elements on JEV prM and E protein expression, recombinant plasmids pCDJE2-7, pCEJE, pRCJE, and pREJE were ini-

TABLE 1. Transient expression of JEV prM and E proteins by various recombinant plasmids in two transformed cell lines

Name	Promoter	Intron	Poly(A)	Ori	Recombinant plasmid	IFA intensity/% positive[a]	
						COS-1	COS-7
pCDNA3	CMV	No	BGH	SV40	pCDJE2-7	3+/40	3+/35
pCBamp	CMV	No	BGH	No	pCBJE1-14	3+/45	ND
pCIBamp	CMV	Yes	BGH	No	pCIBJES14	3+/39	ND
pCEP4	CMV	No	SV40	OriP	pCEJE	2+/4	2+/3
pREP4	RSV	No	SV40	OriP	pREJE	1+/3	1+/2
pRc/RSV	RSV	No	BGH	SV40	pRCJE	1+/3	1+/3
pCDNA3	CMV	No	BGH	SV40	pCDNA3/CAT	–	–

[a] Various cell lines were transformed with pCDNA3/CAT (negative control), pCDJE2-7, pCBJE1-14, pCIBJES14, pCEJE, pREJE, or pRCJE. Cells were trypsinized 48 h later and tested by IFA with JEV HIAF. Data are presented as the intensity (scale of 1+ to 4+) and percentage of IFA-positive cells. pCDNA3/CAT-transformed cells were used as the negative control. ND, not determined. –, negative.

tially tested for the ability to express JEV prM and E proteins following transformation of various mammalian cells. COS-1, COS-7, and SV-T2 cells were transiently transformed with equal amounts of pCDJE2-7, pCEJE, pRCJE, or pREJE plasmid DNA. The SV-T2 cell line was excluded from further testing after preliminary results showed that less than 1% of pCDJE2-7-transformed SV-T2 cells were expressing JEV antigen.

JEV antigens were expressed in COS-1 and COS-7 cells transformed by all four recombinant plasmids, thus confirming that the CMV or RSV promoter and BGH or SV40 poly(A) elements were functionally active. However, the percentage of transformed cells and the level of JEV antigens expressed, as determined by the number of IFA-positive cells and IFA intensity, respectively, differed significantly (Table 1). A significantly higher percentage of pCDJE2-7-transformed COS-1 cells expressed JEV proteins with greater IFA intensity at a level equal to that observed with JEV-infected cells. Cells transformed with the pCEJE, pREJE, or pRCJE vector, on the other hand, showed a lower percentage of antigen-expressing cells as well as a lower IFA intensity. Vectors containing the CMV promoter and BGH poly(A) were selected for further analysis (Fig. 2).

To determine whether the enhanced expression of JEV proteins by the pCDJE2-7 vector was influenced by the SV40 ori, we constructed the pCBJE1-14 vector in which a 2,166-bp fragment containing the f1 ori, SV40 ori, neomycin coding region, and SV40 poly(A) elements was deleted. A chimeric intron was then inserted into pCBJE1-14 to generate pCIBJES14. Plasmid pCIBJES14 was used to determine whether the expression of JEV proteins could be enhanced by an intron sequence. Following transformation, both pCBJE1-14 and pCIBJES14 vectors resulted in cells expressing levels of JEV proteins similar to that observed with the pCDJE2-7 vector (Table 1). These results indicated that expression of the JEV proteins was influenced only by the transcriptional regulatory elements encoded in the recombinant plasmid. Neither the SV40 ori nor the intron sequence enhanced JEV protein expression in the cells used.

Epitope mapping of E protein expressed by a stably transformed cell line constitutively expressing JEV-specific gene products. Authenticity of the JEV E protein expressed by the JE-4B clone was demonstrated by epitope mapping by IFA using a panel of JEV E-specific murine MAbs. JEV HIAF and one irrelevant mouse ascitic fluid were used as positive and negative antibody controls, respectively. Four JEV-specific, six flavivirus subgroup-specific, and two flavivirus group-reactive MAbs reacted similarly with the 4B clone and with JEV-infected COS-1 cells (Table 2).

Detection of JEV E protein secreted by the JE-4B COS-1 cell line. An antigen capture ELISA, employing flavivirus group-reactive, anti-E MAbs 4G2 and 6B6C-1, was used to detect JEV E proteins that were secreted into the culture fluid by the COS-1 cell clone JE-4B. Antigen could be detected in the culture fluid the first day following seeding of the cells with maximum ELISA titers that ranged from 1:16 to 1:32.

Comparison of immune responses in mice vaccinated with pCDJE2-7 genetic vaccine and JEVAX. Plasmid pCDJE2-7 was used as a nucleic acid vaccine to induce an antibody response in mice by immunizing groups of five 3-week-old female ICR outbred mice. Mice were bled at 3, 6, 9, 23, 40, and 60 weeks after immunization, and antibody titers were determined by ELISA or by PRNT. As expected, sera from animals in the pCDNA3/CAT control group did not contain JEV antibody. All animals immunized with pCDJE2-7 and JEVAX seroconverted by 3 weeks after the first vaccination (Table 3). The antibody titers were similar irrespective of the number of doses

TABLE 2. Epitope mapping of E protein expressed by JE-4B, a pCDJE2-7 stably transformed clone of COS-1 cells, with JEV-reactive antibodies[a]

MAb or antiserum	Biological activity of MAb		IFA intensity of cells	
	Specificity	Biological function	JEV infected	4B
MAbs				
MC3	JEV specific		2+	2+
2F2	JEV specific	HI, N	4+	4+
112	JEV specific		4+	4+
503	JEV specific	N	4+	3+
109	Subgroup	HI	2+	1+
N.04	Subgroup	HI, N	3+	4+
201	Subgroup		1+	1+
203	Subgroup		4+	3+
204	Subgroup		2+	2+
301	Subgroup	HI	2+	2+
504	Flavivirus		4+	4+
6B6C-1	Flavivirus		2+	2+
3B4C-4	VEE		–	–
HIAF				
Anti-JEV			4+	3+
Anti-WEE			–	–
PBS			–	–

[a] VEE, Venezuelan equine encephalomyelitis virus; WEE, Western equine encephalomyelitis virus. –, negative.

TABLE 3. Persistence of the immune response in mice (five per group) immunized with pCDJE2-7 or JEVAX

Inoculation[a]	ELISA titer (log₁₀)						PRNT₉₀ titer		
	3[b]	6	9	23	40	60[c]	3	6	9
pCDJE2-7									
1 dose	2.6–3.2	3.8–5.0	3.8–4.4	>3.2	>3.2	2.4, 2.4, 3.8, 4.4	<20	20	40–160
2 doses	2.6–3.8	4.4	3.8–4.4	>3.2	>3.2	2.6, 3.8, 3.8	<20	20–40	40–160
JEVAX, 2 doses	2.6–3.8	4.4–5.0	3.8–5.6	>3.2	>3.2	<2, <2, <2, 4.4	<20	20–40	20–160
pCDNA3/CAT, 2 doses	<100	<100	<100	ND[d]	ND	ND	<20	<20	<20

[a] Three-week-old mice were inoculated i.m. with one or two 100-μg doses of plasmid DNA or twice s.c. with one-fifth of the human dose of JEVAX.
[b] Weeks postimmunization.
[c] Individual serum titers.
[d] ND, not determined.

of pCDJE2-7 or JEVAX given. Mouse serum samples collected 9 weeks after immunization were also tested by Western blotting using purified JEV. Serum specimens from DNA-vaccinated mice, which had reactivity similar to that of JEV HIAF, detected E and prM proteins (Fig. 3). However, mouse serum from JEVAX-immunized mice reacted only with E protein. Comparable ELISA antibody titers were maintained in DNA-vaccinated groups for up to 60 weeks, at which time the experiment was terminated. Only one of four mice in the JEVAX group remained JEV antibody positive at 60 weeks postinoculation. These results demonstrated that one dose of JEV-specific nucleic acid vaccine was more effective in maintaining JEV antibody levels in mice than the commercially available vaccine JEVAX.

Comparison of various nucleic acid vaccine constructs and JEVAX for ability to induce JEV-reactive antibody in different age groups of mice. Similar amounts of JEV protein were expressed by COS-1 cells transformed by either pCDJE2-7, pCBJE1-14, or pCIBJES14. JEV antibody induction by these nucleic acid constructs was compared to results for JEVAX in two different age groups of mice. Three-day-old mixed-sex or 3-week-old female ICR outbred mice, 10 per group, were vac-

cinated i.m. with 50 or 100 μg of plasmid DNA or s.c. with 1/10 or 1/5 of the adult human dose of JEVAX, respectively. Serum specimens were collected at 7 weeks after immunization and tested at 1:400 or 1:1,600 by ELISA. Ninety to 100% of all 3-week-old mice that received pCBJE1-14, pCDJE2-7, pCIBJES14, or JEVAX had antibody titers of ≥1:1,600. However, a significant difference in antibody response was observed in 3-day-old groups that received various vaccines. None of the 3-day-old JEVAX-vaccinated mice had antibody titers higher than 1:400. All 3-day-old mice vaccinated with pCBJE1-14 had antibody titers higher than 1:1,600. Seroconversion of 100% was observed at 1:400 in 3-day-old mice that received pCDJE2-7 or pCIBJES14, but only 60% of both mouse groups were positive at 1:1,600. pCBJE1-14 was the most effective of three DNA constructs tested. The minimum dose of this DNA construct capable of providing 100% seroconversion (1:400 by ELISA) by i.m. immunization in 3-week-old mice was determined to be 25 μg (data not shown).

Protective immunity conferred by the nucleic acid vaccine. Mice immunized at 3 days of age were challenged by the i.p. route at 7 weeks postvaccination with the SA14 strain of JEV (50,000 PFU/100 μl) and observed for 3 weeks. One hundred percent of the animals that received various nucleic acid vaccine constructs were protected. In contrast, only 40 and 30% of mice that received JEVAX and pCDNA3/CAT, respectively, survived virus challenge (Fig. 4). These results suggested that the DNA vaccine could be effective as a neonatal vaccine. In contrast, JEVAX was not as effective in neonatal animals.

FIG. 3. JEV-specific reactivity of prechallenge and postchallenge serum samples obtained from mice immunized with DNA vaccine or JEVAX. Serum specimens collected from the mice used in the experiments represented in Tables 3 and 4 were randomly selected and tested at 1:1,000 dilution by Western blot analysis using purified JEV as the antigen. pCDJE2-7x2-S was the serum from one of the mice challenged at 4 days of age (Table 4). NMAF, 4G2-AF, and JEV HIAF were the mouse ascitic fluids included as normal mouse, E-specific, and JEV hyperimmune controls, respectively.

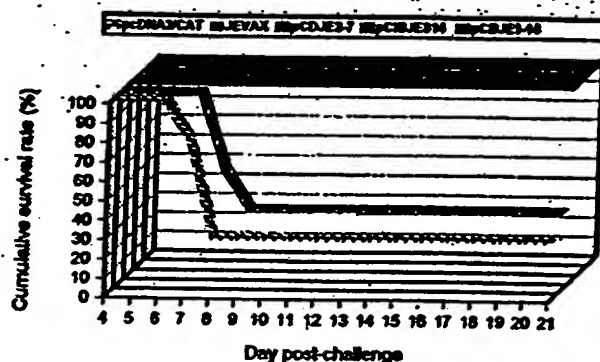FIG. 4. Postchallenge survival rates of mice (10 per group) that were immunized with pCDJE2-7, pCBJE1-14, pCIBJES14, pcDNA3/CAT, or JEVAX at 3 days of age and challenged i.p. with 50,000 PFU of JEV (SA14) 7 weeks postimmunization. A P value of 0.003 was obtained by Fisher's exact test when the survival rate of the JEV DNA-immunized groups was compared with that of the pcDNA3/CAT or JEVAX group.

TABLE 4. Ability of maternal antibody from JEV nucleic acid-vaccinated female mice to protect their pups from fatal JE

Vaccinated mother[a]		JEV-challenged pups			
Vaccine	PRNT$_{50}$	Age (days)	No. of survivors/ total in litter	Avg survival time (days)	ELISA[b]
1 × pCDJE2-7	40	4	0/11	5.27	
2 × pCDJE2-7	80	4	12/12	NA[c]	12/12
2 × JEVAX	20	3	0/16	4.75	
2 × pCDNA3/CAT	<10	5	0/14	4.00	
1 × pCDJE2-7	20	15	5/11	10.0	5/5
2 × pCDJE2-7	40	14	8/12	13.75	7/8
2 × JEVAX	80	13	5/5	NA	5/5
2 × pCDNA3/CAT	<10	14	0/14	6.14	

[a] Mice were inoculated i.m. with one or two 100-µg doses of pCDJE2-7 DNA or twice s.c. with one-fifth of the adult human dose of JEVAX. Serum samples were collected 9 weeks postvaccination for PRNT testing prior to mating with nonimmune male.

[b] Number of JEV ELISA antibody-positive animals (titer ≥ 1:400)/number of survivors. Serum specimens were collected for testing 12 weeks after challenge.

[c] NA, not applicable.

Passive protection of neonatal mice correlated with the maternal antibody titer. Female 3-week-old ICR mice were vaccinated with one or two doses of pCDJE2-7 plasmid DNA (100 µg/100 µl) or twice with one-fifth of the adult human dose of JEVAX. For evaluation of passive protection by maternal antibody, pups were obtained from matings of experimental females with nonimmunized male mice. Pups were challenged by the i.p. route at 3 to 5 or 13 to 15 days after birth with SA14 virus (5,000 PFU/100 µl). Survival rates and average survival time correlated with the maternal neutralizing antibody titers (Table 4). One hundred percent of pups nursed by mothers with a PRNT of 1:80 survived viral infection regardless of the type of vaccine received by the mothers. None of the pups from mothers which received pCDNA3/CAT plasmid DNA survived (Table 4). Partial protection (45% [5 of 11 pups] to 67% [8 of 12 pups]) was observed in older pups that were nursed by the mothers which had serum PRNT titers of 1:20 and 1:40, respectively. However, none of the 3-day-old pups survived virus challenge when the mothers had a serum PRNT titer of 1:20 or 1:40. Maternally transferred antibody can only be detected in the circulation of the young mouse up to 40 days after birth. An appreciable level of maternally derived antibody is maintained in the circulation of the young mouse 24 days or more postpartum (1). JEV ELISA antibody detected in the serum of 97% (29 of 30) of the postchallenge pups at 12 weeks after virus challenge was unlikely to be residual maternally transferred antibody. The presence of JEV antibody in the surviving pups challenged at 3 to 4 or 13 to 15 days of age strongly suggested that maternal antibody did not provide sterilizing immunity to the pups. It also indicated that 3- to 4- or 13- to 15-day-old mice could mount an immune reaction to a live-virus challenge. Partial protection in older pups could be explained by the opportunity to accumulate a large quantity of passive antibody due to the length of nursing time before challenge. One randomly selected postchallenge serum sample also reacted with prM and E proteins by Western blotting (Fig. 3).

DISCUSSION

The flavivirus virion contains a capsid protein (C), a membrane protein (M), and an E protein. The prM MAbs, exhibiting weak or undetectable neutralizing activity in vitro, can

provide passive protection following DEN2 virus challenge (16). However, the E protein plays a dominant role in generating neutralizing antibodies and providing protective immunity in the host. Passive transfer of JEV E-specific neutralizing MAbs has been shown to protect recipients from JEV-induced fatal encephalitis (3, 16, 32, 55). Antigenic and structural analysis using various panels of MAbs has shown that most of the E protein epitopes that elicit virus-neutralizing antibodies are conformationally dependent (9, 40). Coexpression of both proteins as type I transmembrane proteins is essential to maintain proper E conformation and prevent the E protein from undergoing irreversible, low-pH-catalyzed conformational changes (8–10, 19, 50). A 2-kb genomic region, from the internal signal peptide at the carboxyl terminus of C to the transmembrane domain at the carboxyl terminus of the E gene, is essential for expressing authentic proteins. These authentic prM and E proteins are able to self-assemble into virus-like particles in cells infected by either recombinant vaccinia virus or alphavirus vector or in cells transformed by recombinant plasmid DNA (4, 19, 22, 48; Hunt and Chang, unpublished data).

A gene cassette including the elements listed above was amplified from SA14 virus by RT-PCR in the present study. Optimal sequence composition surrounding the translation initiation site (−9 to +4) was incorporated into the 14DV398 amplifying primer (2, 26, 27) (Fig. 1). Recombinant plasmids containing the CMV early gene promoter/enhancer and the BHG poly(A) terminator as transcription regulatory elements expressed JEV proteins with the highest efficiency in three different cell lines. Protein expression and the serological response of mice immunized with DNA vaccine were not influenced by the presence or absence of the SV40 ori or an intron sequence in recombinant plasmids. Virus-specific proteins, secreted into culture medium, could be detected by antigen capture ELISA as early as 48 h after plasmid transformation (data not shown). The authenticity of the E protein produced by the pCDJE2-7 stably transformed cell line, JE-4B, was demonstrated by MAb epitope mapping.

Vaccine potential and characteristics of various eukaryotic plasmids that express flavivirus prM and E proteins are summarized in Tables 5 and 6. All constructs listed had the same transcriptional control elements and similar viral gene cassettes. DEN2 plasmid, which contains prM and 91% of E, is the only exception (Table 6). The JEV DNA vaccine reported in this study is the only construct that stimulated complete protective immunity in mice by a single dose of vaccine given by the i.m. route (Table 5). Sequences surrounding the translation initiation site and the composition of the signal peptide preceding the prM protein are the two major differences among the constructs that may contribute to increasing the vaccine potential of our construct (Table 6). Conserved features of the sequences which flank vertebrate translation initiation sites include a strong preference for purine at the −3 position; a higher frequency of G at positions −9, −6, −3, and +4; and a preference for A or C at positions −5, −4, −2, and −1 (2). Instead of the sequence used in previous publications, the sequence used in our construct was −9 · GCCGCCGCC ATGG, which fits the general criteria listed above. Although less than 1% of eukaryotic mRNA sequences exhibit this sequence, the experimental data have suggested that this sequence provides exceptionally high levels of translation potential (2, 26).

Signal peptides determine translocation and orientation of inserted protein, hence the topology of prM and E. Signal peptide differences in our plasmid construct may account for the efficient translocation and correct topology, thus increasing prM and E secretion. A machine-learning program using neu-

TABLE 5. Vaccine potential of various eukaryotic plasmids that express flavivirus prM and E proteins[a]

Virus	In vitro secretion of EPs	Immunization			Protection from virus challenge	Reference
		Dosage	Route/method	Neutralizing antibody[b]		
JE	Yes	25–100 µg × 1	i.m./needle	Yes (1:20–1:160$_{90\%}$)	100%	This report
	ND	100 µg × 2	i.m./needle	No	Partial	30
	ND	10–100 µg × 2	i.m. or i.d./needle	Yes (1:10–1:20$_{90\%}$)	100%	24
MVE	Yes	100 µg × 4	i.m./needle	ND	Partial	4
	Yes	1–2 µg × 2–4	i.d./gene gun	Yes (80–320$_{50\%}$)	100%	4
SLE	ND	100 µg × 2	i.m./needle	No	Partial	38
CEE	ND	1 µg × 1–2	i.d./gene gun	Yes (1:100–1:1,600$_{80\%}$)	100%	49
RSSE	ND	1 µg × 1–2	i.d./gene gun	ND	100%	49
DEN2	ND	200 µg × 3	i.d./needle	Yes (1:10–1:320$_{50\%}$)	None	17

[a] MVE, Murray Valley encephalitis; CEE, Central European encephalitis; RSSE, Russian spring-summer encephalitis; i.d., intradermal; ND, not done.
[b] Plaque reduction neutralization titer followed by percentage reduction endpoint used in the test.

ral networks trained on eukaryotes (SignalP-NN at http://www.cbs.dtu.dk/services/) was applied to test the efficiency of the prM signal peptide sequence in the different plasmid constructs (34) (Table 6). The most probable location and orientation of transmembrane helices in the prM-E protein were then determined by a hidden Markov model-trained computer program (6 [TMHMM at http://www.cbs.dtu.dk/services/]). SignalP-NN searches correctly predicted the signal peptidase cleavage site of all constructs. However, a considerable difference in cleavage potential (C score, between 0.578 and 1.000) was observed (Table 6). Cleavage potential differences may be influenced by the amino acid composition and length of the h region in various constructs (44).

The TMHMM program correctly predicted five transmembrane helices encoded in the prM-E protein. Significant difference in the probable orientation of the first transmembrane helix was observed in three JEV constructs (Fig. 5). In our pCDJE2-7 construct, the first 12 amino acids of the n region form a short loop in the cytoplasmic side that causes the following h region (transmembrane helix) to be inserted in a tail orientation. Secretion of JEV protein could be detected by antigen capture ELISA in pCDJE2-7 transient expression studies in which less than 5% of the cells were positive by IFA (data not shown). Thus, there is a high probability that prM and E proteins expressed by pCDJE2-7 would be expressed in the correct orientation, as type I transmembrane proteins (Fig. 5A). There is also a high probability that the prM protein of pcDNA3JEME could be expressed as a type II membrane protein with its transmembrane h region inserted in a head orientation because of the absence of positively charged amino acids in its n region (Fig. 5B). Efficient protein synthesis in

conjunction with correct topology of expressed prM and E (Fig. 5A) would most likely enhance EP formation and secretion in transformed cells.

Another characteristic that could explain the excellent vaccine potential of our JEV construct is its ability to produce EPs which have a virus-like polymeric structure that enhances antigenic stability and provides a high-density presentation to antigen-presenting cells, such as macrophages, dendritic cells, and Langerhans cells (5). When DNA is given by the i.m. route, the majority of antigen is expressed by non-antigen-presenting muscle cells. The efficacy of a DNA vaccine is therefore dependent on transfection of antigen-presenting cells or to reprocessing of antigen derived from other cells. Muscle cells transfected by our construct could conceivably synthesize and secrete EPs, which are highly immunogenic and have been shown to elicit good cellular and humoral responses (22, 23).

Genetic JEV vaccine that induced a completely protective immunity in neonatal mice and a maternally transferable protective immunity in young adult mice by a single i.m. immunization was demonstrated in this study. Additional studies are planned to address the effectiveness of a DNA vaccine in overcoming the potential influence of maternally transferred flavivirus antibodies on the induction of JEV antibody in neonatal mice.

Immunization of pigs is a theoretical means of interrupting transmission and amplification of JEV and thereby preventing human infections (43). The JEV DNA vaccine could also be used as a veterinary vaccine in pregnant sows to prevent JEV-induced stillbirth and abortion (51, 53). Maternally transferred antibody could also interrupt piglets as the JEV-amplifying host and thus reduce human infection.

TABLE 6. Characteristics of various eukaryotic plasmids expressing flavivirus prM and E proteins

Virus[a]	Plasmid	Sequence surrounding translation initiation site	Amino acids preceding prM protein[b]		SP potential (C score)[c]	Reference
JE	pCDJE2-7	−9•GCCGCCGCCATGG•+4	MGRKQNKRGGNEGSIDNLASLAVVIACAGA	/MKL	Yes (0.921)	This report
	pJME	−9•GGCTCAATCATGG•+4	MNLASLAVVIACAGA	/MKL	Yes (0.578)	30
	pcDNA3JEME	−9•GAATTCACCATGG•+4	MNEGSIDNLASLAVVIACAGA	/MKL	Yes (0.921)	24
MVE	pCDNA3.prM-E	−9•TGATTTCAAATGT•+4	MSKKRGGSETSVLMVIFMLIGFAAA	/LKL	Yes (0.819)	4
SLE	pSLE1	?	?LDTINRRPSKKRGGTRSLLGLAALIGLASS	/LQL	Yes (0.709)	38
DEN2	p1012D2ME	?	?AGMIDMLIPTVMA	/PHL	Yes (0.646)	17
TBE	SV-PE$_{14}$	−9•GCCGCCGCCATGG•+4	MVGLQKRGKRRSATDWMSWLLVITLLGMTLA	/ATV	Yes (1.000)	48
RSSE	pWRG7077	−9•GTAGACAGGATGG•+4	MGWLLVVVLLGVTLA	/ATV	Yes (0.762)	50
CEE	pWRG7077	−9•ACGGACAGGATGG•+4	MSWLLVITLLGMTLA	/ATV	Yes (0.609)	50

[a] Abbreviations are as given in Table 5, footnote a.
[b] Single amino acid code. Positively charged amino acid is indicated by bold letter. Signal peptidase cleavage site is indicated by /.
[c] Cleavage potential of signal peptide (SP) predicted by SignalP-NN at http://www.cbs.dtu.dk/services (34).
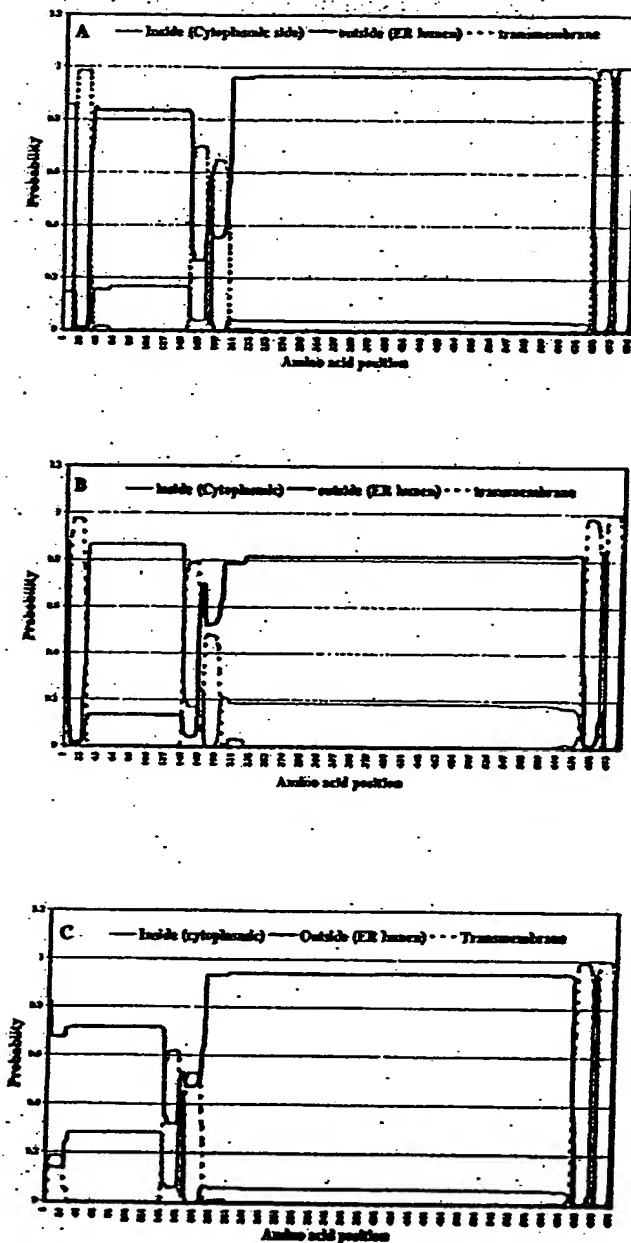
FIG. 5. Graphic representation, generated by the TMHMM program, indicating probable orientations of five transmembrane helices in the prM-E protein expressed by pCDJE2-7 (A), pcDNA3JEME (B), and pJME (C). ER, endoplasmic reticulum.

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Sex Differences in Seoul Virus Infection Are Not Related to Adult Sex Steroid Concentrations in Norway Rats

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Field studies of hantavirus infection in rodents report that a higher percentage of infected individuals are males than females. To determine whether males were more susceptible to hantavirus infection than females, adult male and female Long Evans rats (*Rattus norvegicus*) were inoculated with doses of Seoul virus ranging from 10^{-4} to 10^6 PFU. The 50% infective doses (ID_{50}) were not significantly different for male and female rats ($10^{0.65}$ and $10^{0.8}$ PFU, respectively). To determine whether sex differences in response to infection were related to circulating sex steroid hormones, sex steroid concentrations were manipulated and antibody responses and virus shedding were assessed following inoculation with the ID_{50}. Regardless of hormone treatment, males had higher anti-Seoul virus immunoglobulin G (IgG) and IgG2a (i.e., Th1) responses than females and IgG1 (i.e., Th2) responses similar to those of females. Males also shed virus in saliva and feces longer than females. Manipulation of sex steroids in adulthood did not alter immune responses or virus shedding, suggesting that sex steroids may organize adult responses to hantavirus earlier during ontogeny.

Hantaviruses are negative-sense RNA viruses (family *Bunyaviridae*) encompassing over 20 different viruses that are each carried by a different host species, with rodents serving as the primary reservoirs (18). Field surveys of several rodent species, including brush mice, deer mice, harvest mice, bank voles, and cotton rats, indicate that males are more commonly infected than females (4, 8, 11, 19, 20, 27). Because these studies used serology to determine hantavirus infection, sex differences in infection could reflect either a lack of infection or the absence of sustained antibody production in females. Experimental inoculation of female rodents with hantavirus, however, illustrates that females produce long-lasting, detectable antibody (22). Alternatively, sex differences in hantavirus prevalence may reflect differences in endocrine-immune interactions (15). The extent to which sex steroids affect immune responses against hantavirus infection has not been examined.

In contrast to other rodent species, sex differences in hantavirus prevalence have not been reported consistently among natural populations of Norway rats. Among adult rats, however, males (90%) tend to be infected with Seoul virus more often than females (75%) (7, 10). Seoul virus is hypothesized to be transmitted via wounding, and adult male rats are more likely to be wounded than either females or juvenile males (10). Thus, sex differences in hantavirus prevalence may reflect complex interactions between behavior and physiology. The first goal of this study was to control for sex differences in exposure and determine whether males were more susceptible to hantavirus infection than females. At 70 to 80 days of age, 5 to 10 male and 5 to 10 female Long Evans rats (*Rattus norvegicus*) were inoculated with either 10^{-4}, 10^{-3}, 10^{-2}, 10^2, 10^4, or 10^6 PFU of Seoul virus (strain SR-11) suspended in 0.2 ml of Eagle minimum essential medium (with Earle's salts; Meditach Cellgro, Va.). Seoul virus was obtained from the U.S. Army Medical Research Institute of Infectious Diseases (Ft. Detrick, Md.), where the virus was isolated from neonatal rat brains and

passaged four times in Vero E6 cells. Blood samples were obtained from each animal prior to infection and then 10, 20, 30, and 40 days postinoculation under anesthesia with methoxyflurane vapors (Metofane; Schering Plough, Union, N.J.).

Plasma was used to detect anti-Seoul virus immunoglobulin G (IgG) using an enzyme-linked immunosorbent assay in which microtiter plates were coated overnight at 4°C with gamma-irradiated Vero E6 cells infected with Seoul virus or gamma-irradiated uninfected Vero E6 cells diluted 1:500 in carbonate buffer. Thawed plasma samples, as well as positive control samples (i.e., pooled plasma from rats previously determined to have anti-Seoul virus IgG) and negative control samples (i.e., pooled plasma from Seoul virus-naive rats), were diluted 1:100 in phosphate-buffered saline (PBS)–Tween (PBS-T) with 2% fetal bovine serum and added in duplicate to antigen-coated wells containing either infected or uninfected Vero E6 cells. The plates were sealed, incubated at 37°C for 1 h, and washed with PBS-T, and secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, Md.; alkaline phosphatase-conjugated anti-rat IgG [heavy plus light chains], horseradish peroxidase-conjugated anti-rat IgG1, or horseradish peroxidase-conjugated anti-rat IgG2a diluted 1:400 in PBS with 2% fetal bovine serum) was added. The plates were resealed, incubated for 1 h at 37°C, and washed with PBS-T, and substrate buffer (0.5 mg of *p*-nitrophenyl phosphate per ml diluted in diethanolamine substrate buffer for alkaline phosphatase reactions or tetramethylbenzidine for horseradish peroxidase reactions) was added to each well. Plates were protected from light during the enzyme-substrate reaction, which was terminated after 30 to 45 min by adding 1.5 M NaOH to each well for alkaline phosphatase reactions or 2 N H_2SO_4 to each well for horseradish peroxidase reactions. The optical density (OD) was measured at 405 nm for alkaline phosphatase reactions and 450 nm for horseradish peroxidase reactions, and the average OD for each set of uninfected Vero E6 duplicates was subtracted from the average OD for each set of infected Vero E6 duplicates. Samples were considered positive if the average adjusted OD was ≥0.100. To minimize intra- and interplate variability, the average adjusted OD for each sample

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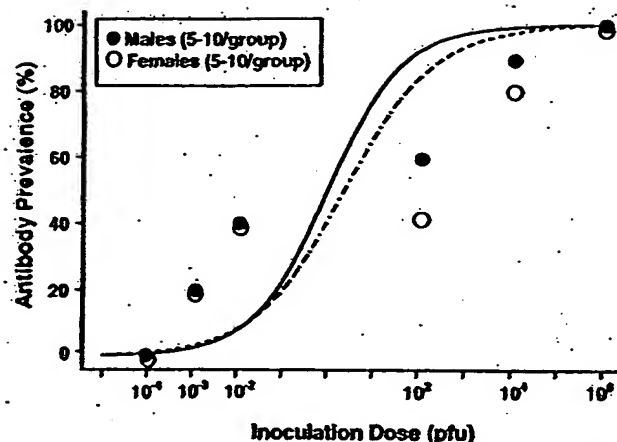
FIG. 1. Antibody prevalence among intact male and female rats inoculated with either 10^{-4}, 10^{-3}, 10^{-2}, 10^2, 10^4, or 10^6 PFU of Seoul virus. Data are presented as percentages of individuals producing detectable antibody (i.e., adjusted average OD $\geq$ 0.100) against Seoul virus by day 40 postinoculation, with the fitted logistic regression curves for both males (solid line) and females (dashed line) included. Equal percentages of males and females seroconverted in response to each dose of Seoul virus ($P > 0.05$ in each case).

was expressed as a percentage of its plate-positive control OD for statistical analyses (9).

Antibody prevalence (i.e., the number of animals with detectable anti-Seoul virus IgG) by day 40 postinoculation was compared between males and females using chi-square analyses. Antibody prevalence was assessed 40 days after inoculation because previous studies illustrate that hantavirus-specific antibody is detectable 15 to 30 days postinoculation (7, 14, 22). Antibody prevalence did not differ between males and females at any of the six doses of Seoul virus ($P > 0.05$). Logistic regression was used to compare the infective-dose (ID) curves and estimate the 50% ID (ID_{50}). The ID_{50} did not differ significantly between males (mean $\pm$ standard deviation, 1.1 $\pm$ 2.0 PFU) and females (7.6 $\pm$ 2.0 PFU) (Fig. 1).

Although the prevalence of males and females that became infected did not differ, studies of other viral infections suggest that patterns of immune responses differ between the sexes and are mediated by sex steroid hormones (1, 15, 29). Thus, males and females may differ because testosterone suppresses and estradiol enhances several aspects of immune function (1, 15, 17, 24, 26, 29). The second aim of this study was to examine whether adult sex steroid hormone concentrations influence immune responses and virus shedding following hantavirus infection. Immunologically, patterns of helper T (Th) cell responses (i.e., Th1 or Th2) differ between males and females, with males exhibiting elevated Th1 responses (i.e., elevated gamma interferon, interleukin-2 [IL-2], and IgG2a levels) and females exhibiting increased Th2 responses (i.e., higher IL-4, IL-5, IL-6, and IL-10 levels) (5, 12, 13). Treatment of males with estradiol and females with testosterone prior to infection with pathogens, such as coxsackievirus, reverses the Th responses, suggesting that hormones can modify immune responses to virus infection (12, 13). To determine whether adult sex steroid hormone concentrations influence immune responses and virus shedding following hantavirus infection, at 70 to 80 days of age 20 male and 20 female rats were bilaterally gonadectomized under ketamine (80 mg/kg of body mass)–xylazine (6 mg/kg) anesthesia (Phoenix Pharmaceutical, St. Joseph, Mo.) and given 2 weeks to recover from surgery. After recovery, 10 castrated males were each subcutaneously implanted with a 30-mm Silastic capsule (inside diameter [i.d.] =

1.47 mm, outside diameter [o.d.] = 1.96 mm) containing 20 mm of testosterone propionate (Sigma, St. Louis, Mo.). The remaining 10 castrated males, as well as 10 intact males, were each implanted with an empty capsule of equal length. Ten ovariectomized females were each subcutaneously implanted with a 15-mm Silastic capsule (i.d. = 1.47 mm, o.d. = 1.96 mm) containing 10 mm of estradiol benzoate (Sigma). The remaining 10 ovariectomized females and 9 intact females were each implanted with an empty Silastic capsule of equal length. Silastic capsule length was based on previous reports that these hormone doses (i.e., the length of the Silastic capsule) are sufficient to maintain physiological testosterone and estradiol concentrations in male and female rats, respectively (25). At the time the Silastic capsules were implanted, all animals received an intraperitoneal inoculation of 10^4 PFU of Seoul virus (strain SR-11) suspended in 0.2 ml of Eagle minimum essential medium (i.e., the ID_{50} from the first experiment). Blood, saliva, and fecal samples were then obtained from each animal on days 0, 10, 15, 20, 30, and 40 postinoculation under anesthesia with methoxyflurane vapors. Saliva samples were collected from anesthetized rats after injecting them intraperitoneally with 2.5 mg of pilocarpine HCl (Sigma) per kg of body mass suspended in 0.9% sterile saline (6). After samples were collected on day 40 postinoculation, animals were killed and seminal vesicles were removed from the males and weighed as an index of long-term testosterone concentrations. All procedures described in this paper were approved by the Johns Hopkins Animal Care and Use Committee (protocol number RA98H536) and the Johns Hopkins Office of Health, Safety, and Environment (registration number A9902030102).

Relative seminal vesicle weights (i.e., corrected for body mass) were higher among intact males (0.282 $\pm$ 0.13 g) and castrated males treated with testosterone (0.326 $\pm$ 0.12 g) than among castrated males (0.095 $\pm$ 0.06 g) [$F_{(2, 29)} = 12.75, P < 0.05$]. Plasma testosterone concentrations in males and estradiol concentrations in females were assayed by radioimmunoassay using the manufacturer's protocols (ICN Biochemicals, Inc., Carson, Calif.). Testosterone concentrations were higher for intact males and castrated males treated with testosterone than for castrated male rats; castrated males treated with testosterone also had higher testosterone concentrations than intact males on days 10, 15, 20, and 30, but not on day 40, postinoculation [$F_{(10, 179)} = 19.30, P < 0.05$] (Table 1). Plasma estradiol concentrations were higher for intact females and ovariectomized females treated with estradiol than for ovariectomized females 10, 15, 20, 30, and 40 days postinoculation; ovariectomized females treated with estradiol also had higher estradiol concentrations than intact females on days 10, 15, 20, 30, and 40 postinoculation [$F_{(10, 173)} = 10.29, P < 0.05$] (Table 1).

Manipulation of testosterone concentrations in males and estradiol concentrations in females did not affect production of antibody against Seoul virus ($P > 0.05$). Overall, males had higher anti-Seoul virus IgG responses than females on days 20, 30, and 40 postinoculation, regardless of hormone treatment [$F_{(5, 353)} = 18.72, P < 0.05$] (Table 2). Male rats also had higher anti-Seoul virus IgG2a responses than females on days 30 and 40 postinoculation despite hormone manipulation [$F_{(5, 353)} = 7.81, P < 0.05$] (Fig. 2A). In contrast, females tended to show higher IgG1 responses than males on days 30 and 40 postinoculation, though this did not reach statistical significance ($P > 0.05$) (Fig. 2B).

Viral RNA was identified using nested reverse transcription-PCR (RT-PCR), and the presence of virus in saliva and feces was used to determine whether virus was shed. Viral RNA was isolated using a guanidine isothiocyanate procedure (3). For

TABLE 1. Sex steroid hormone concentrations[a]

Hormone and group	Hormone concn (mean ± SE) on day postinoculation[b]					
	0	10	15	20	30	40
Testosterone						
Intact males	0.69 ± 0.17*	0.84 ± 0.17*	1.13 ± 0.36*	0.92 ± 0.25*	0.77 ± 0.19*	0.70 ± 0.13*
Castrated males	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T-treated males	0.00 ± 0.00	8.24 ± 0.74*†	6.28 ± 0.91*†	6.62 ± 1.18*†	2.73 ± 0.42*†	0.71 ± 0.28*
Estradiol						
Intact females	25.8 ± 6.81*	27.0 ± 5.57*	20.8 ± 8.39*	25.9 ± 7.78*	38.2 ± 10.1*	55.2 ± 10.2*
Ovx females	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
E₂-treated females	0.00 ± 0.00	166.6 ± 20.6*†	123.1 ± 21.9*†	87.5 ± 8.9*†	162.3 ± 18.8*†	109.7 ± 19.3*†

[a] Sex steroid hormone concentrations in males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E₂]-filled capsules).

[b] Testosterone levels are in nanograms per milliliter, and estradiol levels are in picograms per milliliter. An asterisk indicates that intact and hormone-treated animals had higher hormone concentrations than their gonadectomized counterparts on the corresponding day, based on an analysis of variance ($P < 0.05$). A dagger indicates that hormone-treated animals had higher sex steroid concentrations than their intact counterparts on the corresponding day, based on an analysis of variance ($P < 0.05$).

RNA isolation from saliva, samples were collected from each rat and added to Trizol LS reagent (Life Technologies, Rockville, Md.) at a 3:1 ratio, with RNase-free glycogen (10 µg) added as a carrier. For RNA isolation from feces, approximately 100 mg of feces was homogenized in Tris-EDTA buffer (pH 8.0) and centrifuged at 12,000 × g for 10 min at 4°C; supernatants were collected, incubated with proteinase K (50 µg/ml; Life Technologies) and 0.5% sodium dodecyl sulfate at 50°C for 30 min to digest proteins, and then added to Trizol LS at a 3:1 ratio. To separate, precipitate, and resuspend viral RNA, the manufacturer's protocol was used (Trizol LS; Life Technologies).

For RT-PCR, a 280-bp nucleotide sequence of the SR-11 small (S) genome was amplified using two 20-bp primers, HTN-S4 (5′ GATAGGTGTCCACCAACATG 3′) and HTN-S6 (5′ AGCTCTGGATCCATGTCATC 3′), that amplified positions 979 through 1259 (3). The DNA fragment obtained from the RT-PCR was further amplified using primers HTN-S3 (5′ GCCTTCTTTTCTATACTTCAGG 3′) and HTN-S5 (5′ CCAGGCAACCATAAACATAAC 3′), designed to amplify a 176-bp nucleotide sequence (positions 1031 through 1207). First-strand cDNA was prepared using the GeneAmp RNA PCR kit protocol (Perkin-Elmer, Branchburg, N.J.), incubated in a DNA thermocycler (Techne Genius) at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min, and then held at 4°C. The reaction mixture contained 5 mM MgCl₂, 1 mM deoxynucleoside triphosphates, 1 U of RNase inhibitor, and 2.5 U of murine leukemia virus reverse transcriptase. The positive control was SR-11 RNA isolated

from virus stock, and the negative control was diethyl pyrocarbonate water that was included in the cDNA syntheses and primary and secondary amplifications.

The 280-bp sequence was amplified in a 100-µl reaction mixture containing 20 µl of the cDNA, 0.3 µM HTN-S6 primer, and 2.5 U of polymerase (AmpliTaq; Perkin-Elmer). Reactions were amplified for one cycle at 94°C for 3 min and 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 60 s, followed by 10 min at 72°C. The nested 176-bp sequence was amplified in a 100-µl reaction mixture containing 2 µl of the product of the first DNA amplification, 20 µM HTN-S3 primer, 20 µM HTN-S5 primer, 10 mM MgCl₂, 1 mM deoxynucleoside triphosphates, and 2.5 U of polymerase. Nested-PCR products were amplified using the same cycle series as was used for the primary amplification. The PCR products were electrophoresed on a 4% gel (3% NuSieve plus 1% SeaKem; FMC Bioproducts, Rockland, Maine), stained with ethidium bromide, and examined for bands of the appropriate size. Randomly selected positive PCR products from saliva and fecal samples from males and females, as well as positive and negative control products, were purified using QIAquick (Qiagen, Valencia, Calif.) and sequenced.

Virus shedding in saliva and feces was not altered by hormone manipulation ($P > 0.05$) (Table 3). Overall, more males shed virus in saliva than females 10 days ($\chi^2 = 3.82, df = 1, P = 0.051$) and 30 days ($\chi^2 = 8.19, df = 1, P < 0.05$) after inoculation with Seoul virus (Table 3). The prevalence of Seoul virus in feces also differed between males and females on day 30 postinoculation; more males shed virus in feces than females

TABLE 2. Plasma anti-Seoul virus IgG responses[a]

Group	Anti-Seoul virus IgG response (mean ± SE) on day postinoculation[b]					
	0	10	15	20	30	40
Intact males	0.8 ± 0.6	4.9 ± 3.0	84.0 ± 22.0	106.0 ± 19.0*	332.0 ± 47.0*	342.1 ± 56.0*
Castrated males	1.0 ± 0.7	1.0 ± 1.0	82.0 ± 21.0	106.0 ± 27.0*	280.0 ± 71.0*	387.3 ± 84.0*
T-treated males	1.0 ± 0.7	2.0 ± 0.9	33.0 ± 10.0	108.0 ± 14.0*	314.0 ± 41.0*	426.7 ± 43.0*
Intact females	3.0 ± 1.0	9.0 ± 4.0	36.0 ± 10.0	60.0 ± 14.0	189.0 ± 55.0	219.6 ± 63.0
Ovx females	2.0 ± 0.8	4.0 ± 2.0	7.0 ± 3.0	54.0 ± 16.0	187.0 ± 56.0	209.2 ± 53.0
E₂-treated females	3.0 ± 1.0	8.0 ± 2.0	19.0 ± 6.0	39.0 ± 8.0	178.0 ± 42.0	209.1 ± 39.0

[a] Plasma anti-Seoul virus IgG responses in males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E₂]-filled capsules).

[b] Data are presented as IgG units, in which the mean OD of each test sample was divided by the OD of the positive control sample run on the same microtiter plate. An asterisk indicates that males had higher IgG responses than females, regardless of hormone manipulation, based on an analysis of variance ($P < 0.05$).

A

B

FIG. 2. (A) Plasma anti-Seoul virus IgG2a responses (mean ± standard error) in male and female rats. (B) Plasma anti-Seoul virus IgG1 responses (mean ± standard error) in male and female rats. Blood samples were collected 0, 10, 15, 20, 30, and 40 days following inoculation with Seoul virus. For calculation of IgG2a or IgG1 units, the mean OD of each test sample was divided by the OD of the positive control sample run on the same microtiter plate. Because neither gonadectomy nor hormone replacement had an effect on antibody production, responses from the different treatments groups were collapsed and graphed together. An asterisk indicates that males had higher IgG2a responses than females ($P < 0.05$).

$(\chi^2 = 6.88, df = 1, P < 0.05)$ (Table 3). In general, males shed virus in saliva and feces more consistently than females, regardless of hormone manipulation (Table 3). The PCR product obtained from saliva and feces of males and females was sequenced and verified as Seoul virus DNA.

Sex differences in the prevalence of hantavirus infection have been observed in several natural rodent populations, including deer mice, brush mice, harvest mice, bank voles, and cotton rats (4, 8, 11, 19, 20, 27). In each case, males are infected more often than females. Field studies of Norway rats suggest that sex differences in hantavirus prevalence reflect sex differences in behaviors, like aggression, that increase the likelihood of males being infected (10). High circulating testosterone concentrations increase the probability of engaging in aggressive encounters in several vertebrate species (21). In addition to modulating aggression, sex steroid hormones can

affect immune responses against infection. Studies of viral infections, such as coxsackievirus, suggest that sex differences in both the prevalence and intensity of infection are due to differences in endocrine-immune interactions (12, 13).

Despite the known effects of sex steroids on infection, in the present study, manipulation of adult sex steroids had no effect on immune responses or virus shedding following exposure to Seoul virus. Specifically, males had higher antibody responses and shed virus longer than females, regardless of adult hormone manipulation. Sex steroid hormones affect physiology and behavior at two distinct times during ontogeny (2, 16, 23). During perinatal development, sex steroids cause sex differences in the differentiation or organization of central and peripheral structures. In adulthood, exposure to sex steroids serves to activate preexisting hormonal circuits. The data from the present study may suggest that sex steroid hormones are not involved in hantavirus infection. Alternatively, these data may illustrate that manipulation of activational sex steroids does not alter responses to infection because the hormonal circuitry was organized earlier during development. If sex steroids organize adult responses to infection, then manipulation of neonatal sex steroids should alter adult responses to hantavirus infection.

Regardless of hormone manipulation, males had higher anti-Seoul virus IgG2a responses than females. Recent data from our laboratory indicate that following Seoul virus inoculation, males have elevated IL-2 and gamma interferon concentrations and females have elevated IL-4 responses (S. L. Klein and G. E. Glass, unpublished data). Taken together, these data suggest that males may have higher Th1 responses to hantavirus infection than females. Studies of other viral infections in rodents suggest that females typically have higher Th2 re-

TABLE 3. Virus shedding[a]

Sample and group	No. of virus-shedding rats/total on day postinoculation[b]				
	10	15	20	30	40
Saliva samples					
Intact males	6/11	7/10	6/11	6/11	6/11
Castrated males	4/9	4/9	6/9	5/9	8/9
T-treated males	9/10	7/10	4/10	6/10	7/10
Total males	19/30*	18/29	16/30	17/30*	21/30
Intact females	3/9	6/9	5/9	2/9	2/9
Ovx females	4/10	7/10	2/10	2/10	6/10
E$_2$-treated females	3/10	10/10	3/10	1/10	6/10
Total females	10/29	23/29	11/29	5/29	14/29
Fecal samples					
Intact males	5/11	4/11	4/11	5/11	1/11
Castrated males	6/9	5/9	7/9	4/8	1/9
T-treated males	4/10	6/10	7/10	7/9	1/10
Total males	15/30	15/30	18/30	16/29*	3/30
Intact females	7/9	4/9	4/9	1/8	0/9
Ovx females	9/10	4/10	6/10	2/10	2/10
E$_2$-treated females	6/9	5/10	8/10	2/10	1/10
Total females	22/28	13/29	18/29	5/28	3/29

[a] Virus shedding in saliva and feces from males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E$_2$]-filled capsules).

[b] An asterisk indicates that more males shed virus than females on the respective day postinoculation, based on chi-square analyses ($P < 0.05$).

sponses than males and that this is due, in part, to the effects of estrogens on cytokine production (12). In the present study, females tended to produce higher IgG1 responses than males. In contrast to estrogens, androgens promote differentiation of CD4$^+$ T cells to a Th1 phenotype (12). In the present study, however, castrated and intact males had similar IgG2a responses, suggesting that increased Th1 responses are not contingent on the direct effects of androgens.

High antibody responses in males may indicate that males have more efficient immune responses against infection than females. This outcome seems unlikely given the rapid increase and long duration of virus shedding in males compared to females. Alternatively, males may have higher antibody responses than females because virus replication is increased in males. Higher Th1 responses are associated with increased susceptibility to infections caused by coxsackievirus and Sindbis virus in mice (12, 28). Although quantitative analyses were not conducted, males shed Seoul virus longer than females, suggesting that higher Th1 responses among males may be a consequence of increased virus replication.

In summary, although males and females are equally susceptible to infection with Seoul virus, males shed virus longer and produce higher Th1 responses against Seoul virus than females. Increased virus shedding among males may explain why males are more likely to acquire Seoul virus infection following aggressive encounters among natural populations of Norway rats (10). In the present study, manipulation of adult sex steroid hormones did not alter immune responses or virus shedding following inoculation with Seoul virus. Although sex steroid hormones may not mediate sex differences in response to hantavirus infection, sex differences in infection among adults may be altered by sex steroids earlier during development. Alternatively, sex differences in infection may reflect other neuroendocrine changes, such as differences in glucocorticoids, that may affect responses to Seoul virus infection.

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EXHIBIT 8

Ligand-targeted receptor-mediated vectors for gene delivery

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Gene therapy promises to cure human genetic diseases. One of the main obstacles to fulfilling this promise is in the ability to target a gene to a significant population of cells and express it at adequate levels for a long enough period of time. Viral methods for gene delivery have been studied for a number of years and are effective vectors for gene transfer. The great majority of gene therapy clinical trials currently in progress use retroviruses or adenoviruses. However, there are concerns for their clinical use because of possible risks of mutagenesis, immunogenic side-effects and toxicity. In addition to this, there are other limitations, including the size of gene that can be transferred. Over the last ten years, a new approach has emerged that has increasingly gathered speed thanks to advances in receptor cell biology and antibody production. This method involves ligand-targeted receptor mediated endocytosis (RME) of 'polyplexes'. Here, synthetic complexes are composed of a cell-specific targeting ligand, coupled to a DNA binding element and endosmolytic function. These complexes are able to deliver genes to cells in a receptor-specific manner, without any viral DNA sequences or packaging constraints. There are now many ligand/receptor systems under investigation, each one demonstrating successful gene transfer with a higher level of tissue specificity than viruses can offer. This review describes most of these systems and looks ahead to an era where cell-specific gene delivery may be a main stream gene therapy, treatment modality.

Keywords: polyplexes, receptor-mediated endocytosis, targeted gene delivery, vectors

1. Introduction

There are few areas of biomedical research that have moved as rapidly or so completely captured the imagination of the scientific community as the field of gene therapy. Although in its infancy, gene therapy is a huge commercial business judging by the number of patents filed and of small biotechnology companies starting up, based upon one particular gene therapy technology. This phenomenon surely reflects the enormous potential of this approach for the correction of genetic diseases. There is certainly no lack of ideas, although many are hampered to an extent by the limitations of current technologies. General approaches include replacing a defective gene in diseases such as cystic fibrosis or muscular dystrophy, destroying cancerous or virally-infected cells such as in HIV infection or by promoting a host immune reaction against a disease or infection (for reviews see [1,2]).

When antibodies were discovered, a similar wave of optimism followed. Early this century, Ehrlich hypothesised about 'magic bullets', able to destroy target cells on a specific basis. Monoclonal antibodies brought us a step closer to this dream. Since then, there has been a great deal of research in the area of antibody- or ligand-mediated delivery of drugs, toxins, radioactive isotopes and enzymes, with many promising leads entering clinical trials. Protein engineering has allowed some of these molecules to be improved, and this area is currently one of the most exploited in the biotechnological industry. However, even after almost 30 years of relentless pursuit, nothing has yet delivered such a promise in terms of clinical results.

The delivery of genes encoding various functions greatly expands the range of treatable diseases as well as the types of strategies which can be employed. However, gene delivery remains the major technological stumbling block in gene therapy strategies [3]. Viruses are well suited to deliver genes to mammalian cells by virtue of their infection and replication cycle. Viral delivery is by far the most commonly utilised form of gene transfer vector (reviewed in [4]), with retroviruses being used for many years. Over the last few years, adenoviruses have been developed to overcome some of the limitations of retroviruses and, recently, the two types of viruses have been 'married' to produce a hybrid virus which is able to carry out some of both functions [5]. Retroviruses have the advantages of being potentially low in immunogenicity, with the ability to infect and deliver genes to dividing cells, integrating randomly into the host genome allowing long-term gene expression and heritable transfer. Adenoviruses are able to infect quiescent and dividing cells with much higher efficiency but, being non-integrative, cannot be maintained for long periods of time. They can also provoke a damaging immune reaction.

Whichever viral vector system you chose there are many other drawbacks:

- the risk of secondary malignancies (oncogene activation or tumour suppressor gene disruption) from integrating vectors

- the recombination of disabled viruses could make them infective again

- there is no specific cell specificity, thus allowing non-targeted cells to be infected, a problem

compounded by the heterogeneity of geted antigens

- retroviruses cannot infect non-dividing

- there is an inherent difficulty in produci tres of retroviruses for clinical use, alth(are some strategies being developed to this

- inactivation by host complement (a r sponse to viral infections) in the cases wh fected cells are required to survive, exp toxic viral genes or the immunogenic re cells infected can limit the actual numt transfected

Finally, one of the greatest limitations of vir. livery is in the permitable size of the pack [6].

There is, of course, a great deal of researc: developing viral vectors with improved such as the recently developed lentivir (such as HIV), which can infect non-divid [7] and retroviruses [8] or adenoviruses [9] press targeting ligands on their surfaces. there is also a growing body of research in tive, non-infectious gene delivery methods in [10]). The main examples of these are:

- liposomes (lipid encapsulated DNA) whi rectly with cells to introduce their DNA

- naked DNA (cost-effective injection of into sites of the body receptive to DNA

- ligand-targeted receptor-mediated endc polyplexes

The latter of these is an intensely studied a ing area and is the subject of this review. L like viruses are non-targeted and can cause plement depletion. Their lipophilic nature the ability to transfer DNA to cells with high Naked DNA is simple but non-targetable a from low levels of gene expression and tra efficiencies. RME of DNA by ligands ex highly efficient internalisation pathway ar. ing routes within cells (as do viruses). ligand/receptor complexes gain entry into membrane invagination into clathefin-coa: form endosomes. Various intracellular events result in the release of the ligand ins tosol with some receptors recycling back

surface and some being destroyed by lysosomal degradation (for an example, see [11]).

The initial concept of gene delivery by a non-viral internalised ligand was proposed by Cheng et al. [12], but this group was unable to report successful gene expression. Wu and co-workers [13] exploited the well-studied, liver-specific ASGP-R to successfully deliver and express genes which were attached to one of its natural ligands, asialorosomucoid (ASOR). In effect, the ligand/receptor pathway was being 'hijacked' into additionally transferring a gene. By such routes, thousands of ligands are internalised per second, hence many gene copies can be targeted to cells. Some of these ligand/receptor complexes are highly specific to certain cell-types, opening up the attractive area of *tissue-specific* targeting of genes. Even so, this is not an entirely new delivery pathway for molecules as this has been the primary route for delivering toxins to tumour cells, exploiting the many tumour-associated antigens which are internalised [14]. For gene delivery, the targeting moiety takes the form of an antibody, peptide or natural ligand and the DNA is attached through a DNA binding agent, usually a polycation such as poly-L-lysine, which serves to complex and compact the DNA. These types of gene delivery vectors have been designated 'molecular conjugate vectors' or 'receptor-mediated gene transfer complexes'. It has now been generally accepted that they should be called receptor-mediated 'polyplexes' [15].

2. Receptor-mediated polyplexes

The most basic vector takes the form of a cell-specific ligand and a DNA coupling element. Various elements, such as whole, disabled adenovirus particles, membrane active peptides or translocation domains (see below), have additionally been incorporated to increase levels of gene transfer and expression. Although the exact mechanism of gene delivery is unclear and differs for each receptor, the pathway from the cell surface to the nucleus involves various endosomal compartments resulting in the transport of the DNA to the nucleus for expression [16] (Figure 1). There are many areas to consider when designing such molecules:

- size
- DNA condensation
- route of administration
- nuclease stability

- target sites
- *in vivo* deposition
- cell-binding
- internalisation
- intracellular trafficking

This review aims to address some of these areas. There are many patents in this area (see below), primarily based upon similar ligand-poly-L-lysine conjugates. The payoff for the most successful of these is potentially enormous and will probably result in cross-licensing and collaborative agreements.

2.1 DNA binding element

The way the DNA is attached and held in the complex is crucial to the stability of the vector (*in vitro*-in culture media, *in vivo*-in serum and within the endosomes-once inside the cell) and to the physical size of the complex. Polycationic chains are widely used to neutralise the negative charges of the DNA phosphodiester backbone and allow its condensation into a highly compact form. The more compact and small the complex is, the less chance there is that it will become engulfed by macrophages and be destroyed through the reticulo-endothelial system. The higher the positive:negative charge ratio, the more stable the DNA is to endonuclease degradation as the phosphodiester bonds are less exposed. Additionally, charge neutralisation of the DNA by polycations allows interactions with the negatively charged cell membrane. The polycation/DNA-complex resembles a toroid or 'doughnut' structure (as seen by electron microscopy [17]) with a diameter ranging from 10 - 100 nm, which in some cases can be smaller than a virus. Commonly, Poly-L-lysine is chemically linked to the targeting ligand and this is relatively inexpensive to make. Studies have demonstrated the relationship between poly-L-lysine length and DNA stability or vector function (reviewed in [18]). Poly-L-lysine lengths of 8 residues or less have been shown to result in complexes of up to 3 µm in size, far too large to be taken up by RME [19].

The procedure of complexing the DNA to the polycation has also been studied in an effort to produce better molecules [18]. Generally, DNA and poly-L-lysine spontaneously associate to form a soluble complex. Poly-L-lysine has also been used to compact DNA into liposomes to make the smaller. Kabanov and Kabanov [20,101] have patented variations on polylysine with

Figure 1: Schematic diagram, taken as a summary from many sources, illustrating the general route of gene delivery by receptor-mediated endocytosis. The plasmid DNA, carrying the desired gene is complexed with the ligand-polycation (1) forming a gene transfer polyplex of size 10 - 100 nm (2). This binds to the specific receptor on the target cell (3) which is internalised and forms an endosome (4). Endosomes acidify and cause the break up of the complex (5). Endosomes release their contents into the cytosol, a process dramatically improved by the inclusion of osmolytic agents such as defective adenovirus (6). The DNA, some of which is still bound to the polycation, is localised to the cell's nucleus a process aided by nuclear localisation sequences (7). Gene expression occurs (8), but the DNA is eventually lost as there is no active mechanism to retain it.
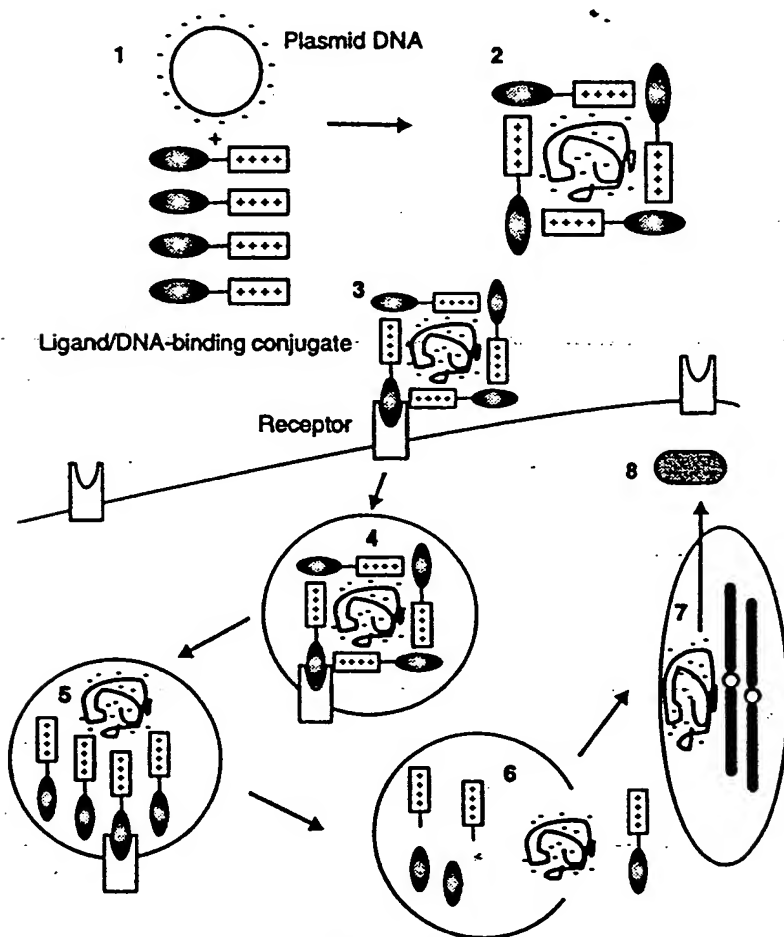
alternative polycation polymers for the use of DNA delivery. Other synthetic polymers such as poly-amino chains with a glucose backbone [21] and poly-ethylemine have been developed. Cationic polysaccharides such as chitosan, which can bind DNA and have lectin specificity, are being studied as dual-function agents for transporting DNA and targeting cells [22]. Modifications such as these alter the kinetics of DNA uptake, but cannot be used in any recombinant approach. Further modifications such as polyethylene glycol (PEG) derivitisation have been patented in a bid to reduce any potential immunogenicity [102].

Commercially available pol preparations tend to be heterog not molecularly defined. There recently, researchers have used occurring DNA binding protein low). Histones and protamines basic, small, compact proteins, · capacity for DNA, but are diffic duce recombinantly. Histone H1 to be superior to the H2-H4 hi: DNA carrier for liver gene delive sperm cell DNA compacting pro mine from humans, was used h tively in the form of an antibody fusion protein produced by man culture [24]. The basic high mol protein (HMG-1) is being studie ternative carrier in recombinant: [25], with expression and activ strated in *Pichia pastoris*. A ba (sequence 'SPKRSPKRSPKR') de the histone H1 sequence [26] an designed sequence ('YKAK$_8$WK the spermidine structure have [27]. Gottschalk *et al.* [27] found quence was ineffective withou ence of an endosome disrupti (see below) and the fully functi was only 10-fold less effective i enovirus vector with 25 - 30% g levels in HepG2 cells. The y binding protein Gal 4 has been ingeniously, despite its lower c DNA (see below [28]). There are ples of where DNA has been dir to the ligand in an approach ten ction' (see below [29]).

DNA intercalating agents have b DNA carriers. Molecules such derivatives [30], ethidium brour dimers [31] and benzoquinone c to DNA, but there are concerns about the the complex *in vivo* due to the DNA condensed and susceptible to nuclease a:

Since there are no packaging requiremen: tation is not an issue with polyplexes. DN up to a size of 48 kbp have been gepor been delivered *via* the transferrin recept low) [32].

The nuclear membrane remains a barrier to gene delivery, as microinjection of DNA into the cytosol results in no expression compared to microinjection into the nucleus [26]. Cells which undergo mitosis after DNA exposure show a higher level of gene expression. Nuclear transport is accomplished by trafficking with a nuclear localisation signal (NLS). These take the form of a short stretch of lysines or argirines (e.g KKKKPRK in the SV40 Large T antigen). These NLSs are transferable with proteins as large as 250 kDa being imported into the nucleus when 'tagged' with this peptide. The lysine-rich sequences used generally serve as satisfactory NLSs as well as DNA binding functions.

2.2 Endosome-disruptive functions

Gene expression levels and periods were initially found to be low by RME delivery of polyplexes and further research showed that the major rate limiting step was the escape of DNA from endosomes and transport to the nucleus (**Figure 1**). This was previously noted for the delivery of toxins in immunotoxins. It was found, then, that co-delivery of replication-defective adenovirus particles greatly increased the rate of endosomal escape. When applied to gene delivery, there was a 200-fold increase in gene expression levels and an increase in levels of transfection to 95% for the cells under study [33,103,104]. This was because the co-internalised adenovirus caused endosome disruption, releasing adenoviral protein (and DNA) into the cytosol. These endosome-disrupting functions are present within the adenovirus coat protein and occur in response to the pH decrease in the endosomes. When the adenovirus is linked to the gene transfer polyplex via an antibody bridge, the rate of gene transfer is improved a further two orders of magnitude [34], perhaps due to the use of a more effective adenoviral NLS. Other methods to link the adenovirus to the gene transfer complex include biotin-streptavidin bridges [35] and chemical linkers [36]. Alternative viruses, such as rhinovirus [37] or naturally-occurring proteins, have endosomal lysis/osomolytic functions, including influenza haemagglutinin (HA) or MS2 phage capsid proteins [105]. Peptides derived from the HA protein have been shown to cause endosomal lysis, but not as effectively as a whole adenovirus. Examples of these include 'GLFEAIAGFIENGWEGMIDGGGC' used in transferrin conjugates [38]. *De novo* designed peptides, based on the amphipathic nature of the haemaglutinin peptides have also been incorporated, such as 'GLFEALLELLESLWELILEA' [27]. Agents such as these have been described in conjunction with alternative DNA delivery vectors to form systems such as SPET-synthetic peptide enhanced delivery [39]

Many toxins have natural endosomal translocation domains which function distinctly to transport proteins across the membrane rather than to lyse the endosome. The 19 kDa diphtheria toxin translocation domain has been used specifically to augment DNA transfer, complexed with poly-L-lysine [40], whereas an existing antitumour *Pseudomonas* exotoxin immunotoxin was modified to deliver a DNA binding protein/plasmid complex, rather than the toxic catalytic domain (see below) [28]. The use of cholera toxin as a delivery and possible translocation domain has also been described as part of a patent for gene delivery to mucosal cells. The B-chains of the cholera toxin multimer may aid translocation across the cell membrane by forming a pore (see below [106]).

In many of the systems studied, the drug chloroquine was used to increase gene expression levels where endosomal processing was involved. Chloroquine is a weak base which neutralises acid compartments. It inhibits hydrolases found in lysosomes and inhibits the fusion of lysosomes with endosomes, thus reducing degradation of their contents and increasing DNA stability.

A different approach to promote release of endosome contents was patented by Berg *et al.* [107]. Here, a photosensitising compound is co-transfected with the DNA, followed by treatment of the cells with light at a certain wavelength. Light-activated, chemically-induced membrane disruption occurs, resulting in endosome release. This can be used for DNA or protein delivery, but may not find wide application *in vivo*.

2.3 Cell-specific ligand

One of the main attractions of this approach is the wide range of ligands/receptors which could be utilised for gene delivery. Examples of systems under study are presented in **Table 1**. This list is growing as fundamental advances in cell biology uncover new receptors and cell determinants. The various groups of ligands will be discussed in the context of the tissues targeted.

Table 1: List of ligands used in receptor-mediated polyplex vectors, the receptors targeted and the cell types expressing th...

Ligand	Receptor	Cell type
Asialoglycoprotein (ASOR) asialofetuin, Galactose/lactose	Asialoglycoprotein receptor (ASGP-R)	Hepatocytes (Parenchymal liver...)
Lactoferrin	Lactoferrin receptor	Hepatocytes (Parenchymal liver...)
Malaria circumzoite protein	Unknown	
α1-Anti-trypsin peptide	Serpin enzyme complex receptor	
Insulin	Insulin receptor	
Reconstituted sendai virus	Unknown	
Transferrin	Transferrin receptor	Malignant cells, glioma epithelia...
Mannose	Mannose receptor	Macrophages...
Lectins	Various	Lewis lung carcinoma...
Epidermal growth factor (EGF)	Epidermal growth factor receptor (EGF-R)	Breast and pancreatic cancer
Folate	Folate receptor	Ovarian cancer
Steel factor	c-kit Receptor	Stem & haematopoietic cells
Peptides containing RGD	Integrins	Endothelial cells
α2-Macroglobulin	α2-Macroglobulin receptor/low density lipoprotein receptor	Liver, intestinal smooth muscle and fibroblasts
Cholera toxin subunit B	GM1 receptor	Mucosal epithelia
Adenovirus pentone base	Unknown	Epithelial cells
Anti-CD3/CD4/CD5/CD7 antibodies	CD3, CD4, CD5, CD7	Lymphomas
Anti-EGF-R antibody	EGF-R	Breast and pancreatic cancer
Anti-ErbB2 antibody	ErbB2/HER2	Breast and pancreatic cancer
Anti-polymeric immunoglobin receptor antibody	Polymeric immunoglobin receptor (pIgR)	Lung epithelial cells
Polyclonal anti-FcR antibodies	Fc Receptor (FcR)	Alveolar macrophages (rat)
anti-? antibody	190 kDa cell surface glycoprotein	Neuroblastoma cells
Anti-CD3 antibody	CD3	Haematopoietic stem cells
Anti-thrombomodulin antibody	Thrombomodulin	Lung endothelial cells
JL1 antibody	Tn Cryptantigen	Cancer, HIV infection, haema... disease
Anti-idiotypic antibodies	Idiotypic antibodies	B-cell lymphomas

3. Systems under investigation

3.1 Gene delivery to the liver

One of the primary targets for gene delivery is the liver, which is the affected organ in diseases such as phenylketouria (PKU), haemophilia and hepatitis infection. The liver is the largest gland in the body, making up about 2% of the body weight. It is central to the metabolism of proteins and lipids, hence is an important commercial target for gene therapy. The ASGP-R is highly expressed on hepatocytes and has become a model receptor for the study of RME and internalisation [11]. The receptor interacts with glycoproteins that have terminal galactose residues. Wu and Wu synthesised a polyplex consisting of the desialated orosomucoid and poly-L-lysine and showed gene transfection of the transformed hepatocyte cell line HepG2 [13]. This was the first example of a successful gene delivered by this method. Reporter gene delivery experiments in vivo showed that 85% of the injected DNA was taken up by the liver by 10 min [41]. A great deal of research has followed, including in vivo gene delivery of albumin to rats with LDL receptor deficiency [42,108]. An average of 1000 copies of the plasmid were found per hepatocyte resulting in a level of 34 μg/ml human albumin in the serum of animals 2 - 4 weeks after injection and partial hepatectomy. Since then, many strategies based on this system have been patented, such as oligonucleotide delivery for antiviral therapy [109] and adenovirus enhancement [110]. Since one-fifth of the cardiac output flows through the liver per minute, this organ is amenable to in vivo gene delivery. Even in the absence of specific targeting, many molecules can be delivered to the liver, although not as efficiently internalised as those that are receptor-directed.

Most of the DNA delivered by this receptor was shown to be degraded, resulting in short-lived (4 days) and low levels of transgene expression. A partial hepatectomy stimulated longer expression, up to 11 weeks in some cases. This was found to be due to increased DNA stability and not due to replication or integration [16]. Further work by others have shown that the intracellular route taken by the DNA complex is not the same as that taken by the free ligand [43] resulting in unpredictable intracellular trafficking.

The human methylmalonyl-CoA mutase gene was delivered to rat liver cells by ASOR-poly-L-lysine [44]. Failure to correct the disease methylmalonic acidaemia is fatal. A staggering 95% of the injected dose accumulated in the liver, once again illustrating how this organ is amenable to this approach. After 6 - 24 h, the blood levels of enzyme increased 30 - 40% over background, although repeated doses were necessary to keep up potentially therapeutic levels.

Lactoferrin has also been used as targeting ligand, in combination with poly-L-lysine. This protein was seen to be better for liver targeting compared to transferrin [45] (whose uses are described later).

An interesting approach to delivering genes to the liver via the insulin receptor was by chemically derivatising albumin, making it positively charged and complexing it with the DNA and insulin [46]. This complex was able to transfect HepG2 cells, but it is difficult to compare this to other systems. Insulin was used to target the liver in a more elegant process patented by the Medical Research Council (UK) [111]. In one example of the invention, a transcription factor is fused to the hormone binding domain of the oestrogen receptor to generate a chimeric transcription factor. This is delivered to the target cell by an antibody. The polyplex is delivered to the same cells by a second antibody of different specificity. The DNA in this polyplex contains the gene (in this case a reporter gene) under the control of the transcription factor delivered by the first antibody. When both complexes are inside the target cell, gene expression will be active in the presence of oestrogen. This results in hormone-responsive gene delivery and expression. The use of two antibodies ensures that cell targeting is specific, as non-specific cells picking up one of the targeting agents will not have active gene expression. This idea is very attractive and adds another level of specificity, but is yet to be backed up with experimental evidence.

Gene transfer to the liver using galactosylated poly-L-lysine showed impressive gene delivery without the need for a partial hepatectomy [47,112]. The factor IX gene, driven by the phosphoenol pyruvate carboxy-kinase (PEPCK) promoter was introduced iv. into rats. The size of the complex was very small (10 - 12 nm) and resulted in the presence of the plasmid DNA episomally for up to 32 days, and the presence of the mRNA and protein for up to 140 days. Gene expression was induced by feeding rats with a high protein/carbohydrate-free diet. Similarly-sized complexes were made using triantennaryoligosaccharides linked to poly-L-lysine, resulting in high levels of gene expression. The high levels of gene expression and the impressive time periods for expression may be related to the size of the small

complexes used compared to the ASOR polyplexes, since the same receptor is targeted. Other carbohydrate-derived targeting complexes have been under study including lactose. Galactose-Histone complexes [23] were 11-times better at delivering reporter genes than ASOR ligands.

The α2-macroglobulin receptor/low-density lipoprotein receptor complex binds and endocytose a wide range of proteins, some of them as a complex with α2-macroglobulin. The α2-macroglobulin receptor is a large complex predominantly expressed on normal liver, smooth muscle cells, neurones and fibroblasts. This receptor was the subject of early studies and subsequent work has shown that the reporter gene, luciferase can be delivered and expressed in these cells by an α2-macroglobulin- poly-L-lysine complex [48]. The wide range of ligands taken up by this receptor make this an attractive target for the delivery of a 'cocktail' of genes, each complexed to a different ligand.

Another receptor complex targeted for gene delivery was the serine protease inhibitor (serpin) enzyme complex receptor (SECR) [49]. This receptor binds to conserved sequences on α1-antitrypsin and other serpins. A peptide based on this conserved sequence was used as a targeting ligand for poly-L-lysine conjugates which resulted in small (18 - 25 nm) complexes. Good levels of gene expression were achieved cells that express the receptor at high levels, such as the liver cells HepG2 and HuH7.

Ding et al. [50] reported that the malarial circumsporozoite protein (that covers the surface of the sporozoite form of the parasite), binds specifically to hepatocytes. Recombinant forms of this ligand have been chemically linked to poly-L-lysine to produce a gene delivery vector. Gene expression via this route is lower than the levels seen with the other methods, but is increased in the presence of adenovirus particles. Gene delivery to other cell-lines such as HeLa, NIH3T3 and K562 was also shown, suggesting a yet undiscovered receptor is being utilised.

Particles, which more closely resemble viruses, such as ligand-targeted liposomes, are being studied. These include asialofetuin-labelled liposomes [51], galactosylated lipopolyamines [52] and reconstituted sendai viruses [53]. These all have liver-cell specificity. Transfection is more efficient than with untargeted liposomes, but the inclusion of NLS sequences or fusogenic peptides does not having a potentiating effect [51].

3.2 Gene delivery to tumour cells

The transferrin receptor is expressed on n thelial cell types but is highly elevated o mours, including gliomas and haem tumours. Many of the early gene delivery e were carried out targeting the transferri which has been a widely studied receptor livery of many agents. Gene transfer to K56 poietic leukaemic cells was achieve transferrin-polycation (poly-L-lysine or conjugate [54,104]. This work pioneered concepts, including the use of virally-de genic peptides as an alternative to whole particles [33]. It was shown that the haen derived peptide from influenza virus was crease gene expression levels 10^2 - 10^4 These 'fusogenic' peptides form m disrupting helices under acid pH condition ing endosomal lysis and gene delivery. Hov 10% transfection rates were seen, comp: 90% seen with whole adenovirus. Also, the seemed to be more toxic than adenovirus

Delivery of genes via the transferrin recep known as 'transferrinfection'. Transferri melanoma cells with the gene for interl sulted in a successful tumour vaccine whic the animals from further tumour challenge variation on the transferrin theme by Scho [56] was to use the high affinity binding streptavidin to cross-link the ligand-targete the condensed DNA complex (Figure 2). F transferrin was used to target the cells, follo addition of biotinylated poly-L-lysine/ poly-L-lysine had 70 residues per chain an ferrin had 1 - 2 biotins per molecule. Gene levels were significant, but the number of fected was not described. This method 100-times more effective than avidin-pe combined with biotinylated transferrin. Ot finity pairs of molecules have been suggest ent to link cell targeting ligands to DNA ca include enzymes/peptide inhibitors an ies/antigens [114].

Neuroblsatoma cells have been found tc 190 kDa cell surface glycoprotein, which i by the monoclonal antibody chCE7. A pc conjugate of this antibody was able to de rase genes at a transfection rate of 1 - 5%, a lower than liposomes, but with 105-fold h of expression [57]. To increase the leve

Figure 2: Avidin or streptavidin has been used to link the DNA carrying element of gene transfer complexes to the targeting element. This can be done directly, where the ligand is biotinylated and cross-links to the DNA complex, or indirectly, where both components are biotinylated and are bridged by streptavidin. Steric constraints suggest that the latter may be more effective for gene delivery.

A   (Strept)avidin-linked poly-L-lysine/
     DNA complex

Biotinylated transferrin

B

(Strept)avidin bridge

poly-L-lysine/
DNA complex

Biotinylated transferrin

Biotin
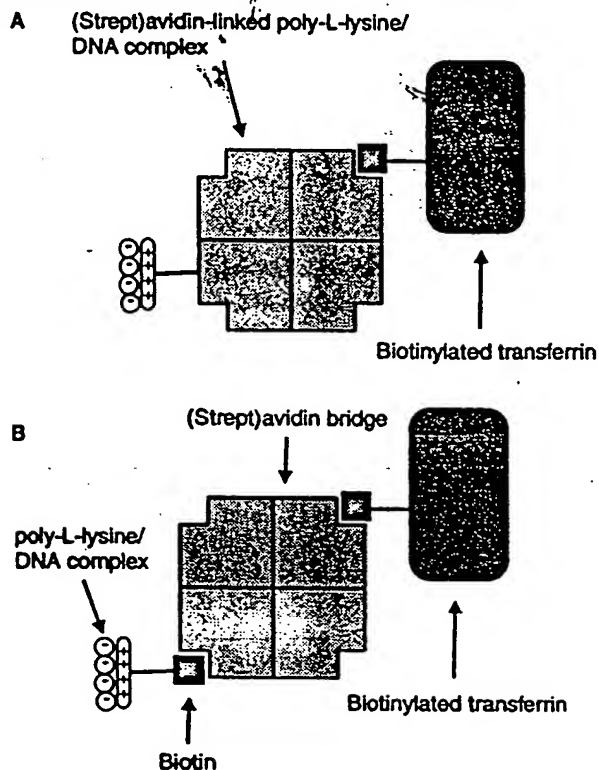
expression using the liposomes, toxic levels of cationic liposomes would have to be administered, whereas poly-L-lysine conjugates are non-toxic. The interferon-γ gene was tested as a more biologically relevant gene and it was found that HLA expression increased to higher levels than would have been achieved if 1000 IU/ml of pure exogenous IFN-γ was applied. Thus, targeted gene expression of IFN-γ proved much more effective and resulted in cytotoxic T-cell responses *in vitro*.

The normal liver expresses the epidermal growth factor receptor (EGF-R), but this receptor is highly elevated in many squamous cell carcinomas including breast and lung. The monoclonal antibody B4G7, which is internalised by EGF-R, was used successfully to deliver the CAT (chloramphenicol acetyl transferase) gene to tumour cells [58,115]. Further work showed that this system was able to deliver a suicide gene, herpes simplex virus thymidine kinase [59]. The transfected cells were 10-times more susceptible to

the prodrug, gancyclovir, which resulted in 70% cell-killing, but these results are still a long way behind those achieved by viral delivery of prodrug-activating enzymes (VDEPT) (reviewed in [60]).

The natural ligand, EGF, has been used in a streptavidin-poly-L-lysine/biotinylated EGF system deliver the β-galactosidase gene aided by replication-defective adenovirus [61,116]. A four-fold improvement was seen in the presence of chemically linked replication defective adenovirus, with 14 - 99% cell transfection rates observed. Whether the proliferative effects of the EGF had any role in the transfection rates is not known, but this type of approach is promising for the delivery of p53 or k-*ras* gene to correct some lung cancers.

EGF-labelled liposomes have been used as an alternative to poly-L-lysine as the DNA carrying agent [62]. These targeted liposomes give only a 2-fold increase in the level of gene expression compared to non-targeted liposomes, *in vitro*, with high transfection rates (6 - 8%). However, how liposomes will behave *in vivo* needs to be addressed.

Fominaya and Wels suggested that, in general, effective gene delivery vectors would require about 10^5 - 10^6 adenoviral particles per target cell, or 10 - 100 μM fusogenic peptide, 50 - 100 μM chloroquine and a nuclear localisation sequence (usually sufficed by poly-L-lysine). An ingenious extension to their immunotoxin research [28,117] was to replace the catalytic domain of *Pseudomonas* exotoxin A immunotoxins with the DNA binding domain of the Gal 4 transcription factor (from yeast). Therefore, instead of delivering a toxin to the target cells, a gene could be delivered by the same route. The cell binding domain remains the recombinant single-chain Fv fragment against the erbB2 receptor, the immunotoxin's translocation domain can substitute for the adenovirus particles and the Gal 4 domain contains the DNA binding/NLS motifs (Figure 3). The Gal 4 fragment does not have as high a capacity for DNA as poly-L-lysine, as it binds through a sequence-specific zinc finger interaction rather than the non-specific electrostatic interactions of the latter. Any remaining negative charge was compensated by additional poly-L-lysine. Like the *Pseudomonas*-derived immunotoxins, these 'all-in-one' molecules express to high levels in *E. coli* and a high yield of recombinant product can be recovered. The added attraction of this system is that the amount and type of DNA which is bound in the complex can be controlled by the number of repeats of the Gal 4
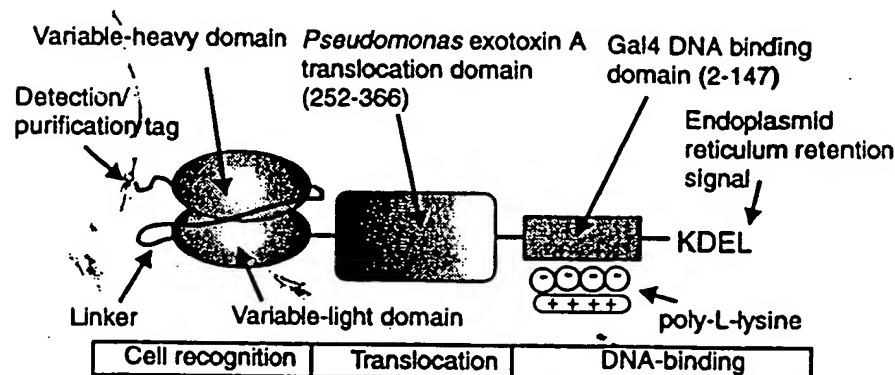
Figure 3: The modular ; recombinant gene transfe was demonstrated by Fo. Wels [28,117]. Here recognition domain is a antibody, linked to a transle *Pseudomonas* exotoxi: DNA-binding domain fro' transcription factor. Tl tetrapeptide sequence retention of the comp endoplasmic reticulum, n amount of protein lost tc degradation and targetin compartment it translocate retrograde trafficking.

recognition sequence. The more repeats, the more protein can bind to the same plasmid molecule. This may provide a way of controlling the kinetics of plasmid uptake. The Gal 4-DNA interaction is highly specific and of high affinity, possibly stabilising the DNA on route to the nucleus. Cos-1 cells were successfully transfected with this complex.

The ErbB2 (Her 2) receptor is restricted more to tumours than the EGF-R, and has been the subject of targeting with a humanised antibody (rhumAbHer2)-poly-L-lysine conjugate [63]. Gene delivery was almost 200-fold higher than with an irrelevant antibody. An NIH3T3 cell line transfected with the receptor as well as carcinomas were able to take up the gene specifically.

Glycoproteins of the mucin family, have been studied as targets. The Tn cryptantigen, which is expressed in cancers, haematopoietic disorders and on the HIV virus coat glycoprotein, is expressed in the model 'Jurkat' cell-line. This is due to a defect in the Tn-processing galactosyl-transferase. Gene delivery through this receptor has been accomplished using the 1E3 monoclonal antibody linked to poly-L-lysine [64]. Adenoviral particles increased transfection efficiency to 60%. Treatment of the cell with sialidase (which removes sialic acid and exposes more Tn antigen) increased gene delivery levels; competing GalNAc reduced gene expression. The transferrin receptor is also expressed on this cell-line and a direct comparison showed the Tn-antigen mediated system to be better. There is a 10-fold higher level of Tn antigen on Jurkat cells compared to transferrin, but gene delivery was 40-fold higher. However, the presence of multiple epitopes on a single Tn-protein may account for the better targeting.

Anti-idiotypic antibodies represent one of t tumour-specific antibodies, with successfu of their targeting evident in cancer immuno natural development to this was to use thes as targets for gene delivery to B-cell lympi Poly-L-lysine conjugates of anti-idiotypic show highly specific gene delivery.

Various lectins have been tested as possi: for RME endocytosis of DNA by tumour c cell surface. Concavalin A was found to when biotinylated, and linked to the pc conjugated antibiotin antibody [66]. Rec these lectins are over-expressed on mai such as Lewis lung carcinomas.

Small molecules can also be taken up by ceptors by a process called 'pinocytosis'. Fc tors are often over-expressed on ovarian ti and folate-labelled liposomes carrying pc condensed DNA have been successful in genes [67] and antisense oligonucleotides tumour cells. These complexes are relative: liposomes, 74 nm in diameter. Antisense E(nucleotides were able to inhibit EGF-recep sion in these cells and cause a 90% reduc growth, suggesting that significant amoun nucleotide DNA can be delivered by this

3.3 Gene delivery to lymphocytes

The CD3 receptor is expressed on 95% of level of about 10 - 40,000 molecules per ce genic binding results in a rapid rate of e: 420,000 molecules over a period of 24 h. (OKT3, WT32 and UCHT-1) against the cule, conjugated to poly-L-lysine were use a CMV-driven luciferase gene to T-cells SPET/AVET system. Up 50% transfection seen in Jurkat cells, increasing in the p

chloroquine and membrane-active peptides (10- to 100-fold). *In vivo*, 1000 - 2000 units of interleukin-2 were expressed from transfected Jurkat cells, which peaked at 24 h. Peripheral blood lymphocytes were transfected at a lower rate (5%).

A study compared the effectiveness of gene delivery to lymphoid cells *via* the CD3, CD34 and surface immunoglobulin receptors, using monoclonal antibodies in a technique called 'antifection'[29]. Although the transferrin receptor is prevalent on lymphoid cells, delivery by this route compared to these other receptors is about 1000-fold less effective. In this system, there is no DNA condensing agent as the plasmid molecule is directly coupled to the antibody. *In vitro* transfection results were not as good as conventional approaches, with 0.1% transfection rates at best. However, *in vivo* on spleenocytes, impressive 1 - 7% transfection rates were seen as detected by β-galactosidase expression and neomycin resistance. This compared well to the poly-L-lysine mediated system above [39]. There was no discussion about the size of these complexes or the stability of the DNA. A high affinity antiCD5 antibody (T101) has also been used, linked to poly-L-lysine to deliver a reporter gene to lymphocytes [69].

Steel factor is peptide ligand, which binds to the c-*kit* receptor on primitive haematopoietic stem cells. Streptavidin-conjugated poly-L-lysine/DNA complex was targeted to cells by biotinylated steel factor [70]. After 2 h incubation, the maximal transfection efficiency approached 90% with maximal gene expression after 30 h. The gene expression was improved almost 10-fold by the addition of adenovirus to promote DNA endosomal escape. In this example, the strategy allowed the mixing of any biotinylated ligand to the DNA complex to deliver genes to a wider range of cells. Transferrin was used to illustrate this. The high efficiency of gene delivery could be used for the purging of bone marrow *ex vivo* by the delivery of suicide genes.

Gene delivery directly through the CD3 receptor has been shown to result in low levels of expression attributed to the induction of TNFα-mediated apoptosis, caused by binding the CD3 receptor [71]. It was shown that this effect could be counter-acted by the inclusion of anti-TNFα-antibodies during transfection, resulting in increased proliferation rates of transfected lymphocytes.

Bispecific antibodies (bsAbs) present an alternative way to deliver genes to lymphocytes. This is exemplified by the use of an antiCD3/anti-FLAG bsAb to target FLAG-peptide bearing adenovirus to CD3-expressing cells [72]. However, this is still re-direction of infectious virus.

In a study to find alternative ways to deliver toxins without the problems of toxin immunogenicity, Chen *et al.* [24] extended the ligand-cation polyplex research area into the area of recombinant fusion protein construction. They created a recombinant antibody (Fab) against the HIV coat protein gp120, fused to the human DNA binding protein protamine. Recombinant fusions have the advantages of being a homogeneous species of purified molecule, which can be rationally designed using the tools of protein engineering. This completely human-derived fusion protein polyplex was able to deliver the gene for a toxin, *Pseudomonas* exotoxin A, resulting in cell-targeted cytotoxicity. Highly cationic polypeptides are notoriously difficult to produce recombinantly, making recombinant production of such gene therapy vectors troublesome, although the advantages are attractive.

Lymphocytes can also be targeted by interleukin-2, as has been seen in tumour targeting. Gene delivery fusion proteins (GDFPs) based on IL-2 and Gal4 (as the DNA binding domain) have been patented and shown to localise plasmid DNA to cells, but no expression was reported [118]

3.4 Gene delivery to macrophages

Alveolar macrophages play a role in lung homeostasis and pathogenesis of disease. Cognate polyclonal antibodies have been produced against the Fc-receptor of these cells and used chemically conjugated to a 30 kDa poly-L-lysine chain [73]. Gene expression in purified alveolar macrophages of β-galactosidase was 5-times that of the background and increased further in the presence of chloroquine. Gene delivery was specific as it was competed by unconjugated antibody and did not transfect Fc-R-negative cells.

Peripheral blood macrophages possess mannose receptors which have also been targeted for gene delivery [74]. Mannosylated poly-L-lysine was the delivery vector and expression was short-lived, peaking at 4 days and dying out 16 days. Expression was confirmed to be confined to macrophages as the localisation of non-specific esterase enzyme marker correlated with gene expression. The DNA complexed was well-neutralised with about a 1:0.9 ratio of DNA:poly-L-lysine. The chain length was about 100

Table 2. Some examples of promoters that could be used in conjunction with targeted gene delivery, and the disease suitable.

Disease/tissue	Gene	Promoter
Insulin-dependent diabetes mellitus/pancreas β-cells	Insulin	Insulin promoter
Hepatoma/liver	Cytotoxic	α-Fetoprotein
Metabolic diseases/liver	Factor VIII, IX, PKU	Albumin
Muscular dystrophy/muscle	Dystrophin	α-Actin
Lymphomas/B-cells	Cytotoxic	Immunoglobulin heavy chain
Cancer/breast	Cytotoxic	Erb B2
Melanoma/melanocytes	Cytotoxic	Tyrosinase
Lymphomas/T-cells	Cytotoxic	T-cell receptor
SCID/T-cells	Adenosine deaminase	T-cell receptor
Colorectal cancer/GI tract	Cytotoxic	Carcinoembryonic antigen

residues with about 1% being glycosylated. Electron microscopy showed the whole complex to be a favourably small 10 - 20 nm in diameter. Such vectors could be used to treat reticulo-endothelial storage diseases such as Gaucher's disease.

Antisense oligonucleotides have also been delivered by this route [75], with the ablation of TNF-specific mRNA. This adds support to the view that this method may be able to achieve significant levels of delivered oligonucleotide.

3.5 Gene delivery to epithelial cells

Respiratory epithelial cells express the polymeric immunoglobulin receptor (secretory component) which is involved in transporting antibodies across mucosal layers. An antibody (Fab)-poly-L-lysine complex was used to deliver genes to epithelial cells *in vitro* [76] and *in vivo* [77]. Injection of the complex into rats resulted in high levels of gene expression in the surface epithelium and submucosal glands of the lung and with some expression in the liver, demonstrating excellent targeting. The level of expression lowered upon multiple injections due to the illicitation of an-tirabbit antibodies [78]. The important findings were that there were no anticomplex or anti-DNA antibodies raised in response to the treatment. There was no activation of complement, although it is thought that free poly-L-lysine can activate complement by the

alternative pathway. Airway epithelial cell a good target for gene delivery as transfecticies in the region of 10% may be enough to symptoms cause by cystic fibrosis disease

The gastrointestinal (GI) epithelium is an target in terms of absorption/secretion an disorders and malignancies. The transferr can be used to deliver genes to GI epithelicolon carcinoma cells [80]. High levels of of the serum protein α₁-anti-trypsin were opening up the possibilities of correcting a rum protein deficiencies by this route as liver.

Lectins can also be used to target epithei well as tumour cells. Concavalin A linked proteins have shown the most promising this route [81]. The natural affinity of adeepithelial cells has been exploited in the us rus fibre and pentone coat proteins as targ genic proteins [82].

Epithelial cells are specifically bound by protein produced by some pathogenic bac of their infection process. Invasin has beetargeting ligand in conjunction with the binding protein to achieve specific report pression [83]. In another example of derived targeted vehicles, the cholera toxi

composed of a AB5 hexamer, has been used to deliver DNA to the gastric epithelial cells normally attacked by the toxin itself. The cell binding domain (B subunit) which targets the GM1 glycolipid receptor on mucosal epithelia, was linked to poly-L-lysine as used to deliver functional cystic fibrosis gene mRNA, reducing cystic fibrosis symptoms [106].

3.6 Gene delivery to other cell types

A variety of ligands were screened for most effective binding to myogenic cells. Transferrin was selected and used to deliver genes to these cells opening up the possibility of targeting a correction for Duchenne muscular dystrophy [84].

Tissue-specific lung gene delivery to lung endothelial cells has been achieved using a monoclonal antibody-poly-L-lysine conjugate. The antibody recognised the cell surface thrombomodulin [85]

Integrin research is expanding rapidly as they are found to be involved in a great many cell-cell interactions. These are particularly embryogenesis, tumour metastasis, wound healing and T-cell function. Cyclic peptides containing an integrin recognition sequence 'Arginine-Glycine-Aspartate' (RGD) has been used, fused to a poly-L-lysine chain [86,87,117]. The entire targeting construct was chemically synthesised, making this a simple molecule to make and develop. High levels of gene expression were seen in a variety of cell lines bearing the receptor.

A general cell targeting construct was created by fusing the immunoglobulin-binding domain of protein A to the coat protein of sindbis virus [88]. Cell targeting is achieved by incubating the chimeric virus with a cell-specific monoclonal antibody. This allows cell specificity to be altered easily by changing the antibody.

4. Conclusions

Presently, this approach to gene delivery is much less efficient than viral gene delivery. However, under optimal conditions, enough gene product may be produced to give a therapeutic benefit (e.g., suppress a phenotype or destroy a tumour). With current technologies, it is very likely that multiple doses will be needed to maintain adequate expression levels. If the complexes are not immunogenic (i.e., human proteins used), this may be a viable option, perhaps more desirable in that a controllable, safer treatment modality is achieved. Therefore, the potential drawbacks are

compensated by the significantly lower risk levels associated with this method.

It is difficult to compare the efficiency of different gene delivery systems, especially between those that target different receptors as each delivery route is different. Different reporter genes are used and groups using the same gene, e.g., luciferase, describe different ways of presenting the results. Nevertheless, one can get an idea of how good a system is by measuring the time of gene expression, the percentage of cells transfected and, ultimately, the curative effects in an animal model *in vivo*. Although better targeted, by these measurements, they lag behind viral methods.

However, the practically unlimited size restraints on the size of DNA deliverable by these receptor-mediated polyplexes can give these systems a major advantage over viral targeting. Genes for large proteins, sets of genes or genes with complex regulatory sequences could easily be accommodated. The expression of integrated genes tends to be higher for those that contain the correct intron structure to allow proper processing. This is true for transgenic animal gene expression [89].

This is certainly a growth area in gene therapy. Many of the targeting constructs are similar in basic structure (ligand-polylysine/polycation-adenovirus/fusogenic peptide), which has been demonstrated to be very effective. Many of the patents in this area describe the novel invention (e.g., a specific ligand or coupling method), followed by a description of general applicability. Therefore there seems to be a large degree of overlap between patents.

Research into tissue-specific targeting of tissues such as the liver, bone marrow stem cells and macrophages is well advanced, with good prospects for clinical testing. Tumour targeting of genes is also progressing well, basically following the same lines of receptor targets as previous immunotoxin research. Genetic delivery of toxins to tumours may prove to be more effective than immunotoxins.

5. Expert opinion

Gene delivery by ligand targeted receptor-mediated endocytosis of polyplexes should find its way into some main line gene therapy treatment schemes by virtue of its superior specificity, lower risk and reduced size limitations. However, in order to achieve the levels of gene transfection and expression seen

with retroviral vectors, further advances need to be made in fields such as mammalian artificial chromosomes [90]. Potentially, once genes are specifically delivered, they may be maintained for long periods of time in a way analogous to bacterial plasmids or artificial chromosomes (BACs) or yeast artificial chromosomes (YACs)

The powerful combination of cell-specific targeting *via* receptors and promoter specificity may allow an even higher degree of specificity resulting in 'super--specific targeting' of genes to cells with very little non-targeted expression. For example, the targeting of genes to the liver *via* the asialoglycoprotein can be combined with the use of the liver-associated albumin promoter, or the genes delivered to tumour cells through the erbB2 receptor may be placed under the transcriptional control of the erbB2 promoter. There is already evidence of this approach being used [42], but there are many more possible tissue or tumour specific promoters which could be utilised in this way (Table 2).

Library selection techniques such as phage display will ultimately yield new tissue or tumour-specific antigens to expand the options available for cell targeting. Organ-specific peptides have been isolated using elegant cell-selection strategies [91] or *in vivo* panning [92]. The more ligands we have at our disposal, the better options we will have for specific targeting. Combinations of specificities may overcome antigen heterogeneity problems.

Finally, advances in heterologous gene expression systems will also expand the options available in the construction of such delivery vectors. The ability to produce highly basic proteins, which are notoriously difficult to express recombinantly at high levels, combined with protein engineering and rational design will provide the researcher with more advanced tools for constructing effective gene delivery agents, such as those which are activated to bind tumours when processed by tumour-derived matrix metalloproteinases (as seen for recent retrovirus constructs) [93].

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EXHIBIT 9

Targeted vectors for gene therapy

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ABSTRACT   Successful gene therapy requires not only the identification of an appropriate therapeutic gene for treatment of the disease, but also a delivery system by which that gene can be delivered to the desired cell type both efficiently and accurately. Reductions in accuracy will inevitably also reduce efficiency since fewer particles will be available for delivery to the correct cells if many are sequestered into nontarget cells. In addition, the therapy will have net benefit to the patient only if gene delivery is sufficiently restricted such that normal cells are left unaffected by any detrimental affects of bystander cell transduction. Here we review how currently available delivery systems, both plasmid and viral, can be manipulated to improve their targeting to specific cell types. Currently, targeting is achieved by engineering of the surface components of viruses and liposomes to achieve discrimination at the level of target cell recognition and/or by incorporating transcriptional elements into plasmid or viral genomes such that the therapeutic gene is expressed only in certain target cell types. In addition, we discuss emerging vectors and suggest how gene therapy delivery systems of the future will be composites of the best features of diverse vectors already in use.— Miller, N., Vile, R. Targeted vectors for gene therapy. *FASEB J.* 9, 190–199 (1995)

Key Words: targeting · retrovirus · adenovirus · liposome

THE IDENTIFICATION OF THE UNDERLYING genetic defects has recently made gene therapy an attractive treatment option for a wide variety of diseases. However, there is a corresponding requirement to produce vector systems that can deliver therapeutic genes to the appropriate target cells either in vivo or ex vivo. These systems must be both *efficient* and *accurate*. The range of different diseases amenable to intervention by gene therapy means, however, that no single delivery system is likely to be universally appropriate. For instance, the requirements of gene therapy for cystic fibrosis are greatly different from those of cancer. In the former case, only a certain proportion of a localized population of cells needs to be targeted with a single corrective gene; by contrast, cancer gene therapy usually involves the targeting of all of a diffusely spread population of cells, with the ultimate aim of killing rather than correcting them. Hence, the stringency with which the therapeutic gene needs to be accurately delivered can vary greatly. Expression of a copy of the cystic fibrosis transporter gene in nontarget cells is likely to be much less toxic than inadvertent expression of cytotoxic genes, aimed at cancer cells, but expressed in normal bystander cells.

Here, we review the progress in targeting gene delivery systems to specific target cell populations and look forward to the areas of research that will bring developments for the future. Unfortunately, improvements in the accuracy of a vector often compromise its efficiency, and vice versa. Nonetheless, it is clear that the technology now exists to incorporate specific targeting features into most of the currently available delivery systems. These may be at the level of *1)* target cell surface recognition, by manipulating the surface recognition components of viruses and liposomes; or *2)* target cell transcriptional restrictions, by incorporating transcriptional elements into plasmid or viral genomes such that the therapeutic gene is expressed only in certain target cell types.

The ultimate aim for the vectors of the future is to include these and other targeting opportunities within the same vehicle. In all probability, this will involve the incorporation of the most beneficial features of a variety of viral and nonviral systems into a single hybrid vector specifically custom built for each individual therapeutic situation.

TARGETING OF GENE THERAPY VECTORS AT THE LEVEL OF THE CELL SURFACE

Retroviral vectors

A primary determinant of retrovirus infectivity is the interaction between specific receptors on the host cell surface and glycoproteins (Env) on the lipid envelope of the retroviral particle. Ideally, targeted retroviral vectors for human gene therapy would use safe recombinant genomes and packaging lines from wild-type retroviruses that naturally display envelope proteins with the required tropisms. However, few naturally occurring retroviral infections are strictly limited to one cell type (1), and of the known receptors for retroviruses, only the HIV-1/SIV receptor CD-4 (2) is of relatively restricted distribution. Attempts have been made to produce vectors and packaging lines from HIV (3). However, HIV is a complex retrovirus that requires a number of self-encoded autoregulatory proteins, and this complicates the construction of stable packaging lines. Nevertheless, the principle of a recombinant HIV genome as a gene vector for CD4+ cells has been demonstrated (3). However, vectors carrying HIV-1 *env* sequences would have to be used with extreme caution as the HIV-Env protein itself may be neurotoxic (4) or even immunosuppressive.

Most recombinant retroviral vectors and packaging lines produced so far have been based on murine leukemia viruses (MLVs)[2] (5). There are five recognized MLV groups (1) as

defined by tropism, of which the most useful for gene delivery purposes have been the ecotropic strain (MLV-E), which infects virtually all rodent cells, and the amphotropic strain (MLV-A), which infects practically all mammalian cells. Packaging lines have therefore been created to allow production of retroviral vectors with host ranges that are either ecotropic or amphotropic, respectively (5). It is likely that all retroviral vectors suitable for human gene therapy in the near future will be based on such recombinant MLV genomes because they are well characterized with regard to safety and efficiency. For targeted retroviral vectors, then, the problem is either to restrict the promiscuous tropism of amphotropic particles or to confer upon ecotropic particles a limited human cell affinity. This could be done either by: 1) genetic manipulation of the producer line such that amphoor ecotropic Env is replaced by a different viral or nonviral protein having the required affinity; 2, directly ngineering a particular affinity into Env; or 3) molecular conjugate approaches, in which ligands are coupled to the outside of the retroviral particle.

Replacement of Env: retroviral pseudotypes

The facility (5) with which *trans-* and *cis*-acting functions can be separated in MLV packaging lines allows easy experimental manipulation of the *trans*-acting function responsible for cellular tropism, namely, Env. This raises the possibility of replacing one viral *env* with that of another, thereby creating a hybrid producer line that generates "pseudotyped" viral vectors with a tropism conferred by the replacement *env* (Fig. 1). Phenotypic mixing has been used for many years as a tool to study receptor interactions (see ref 1 for a review); however, efforts have recently been directed at precisely replacing *env* and producing not envelope mixtures but vector populations exclusively displaying a novel tropism (1, 6). Such hybrid formation in general seems to occur more

efficiently between closely related viruses. For instance, a recombinant MoMLV genome can be rescued by C-type viruses but not by HTLV-I or D-type viruses (7). However, provision of homologous or more closely related Gag proteins in some cases relaxes phenotypic restrictions on efficient pseudotyping of vector genomes with exogenous Env; for instance, an MoMLV vector can be packaged inside HTLV-I.(8) envelopes when MoMLV *gag-pol* are supplied in *trans*. Similarly, HIV has been given an extended host cell range by pseudotyping with the unrelated viruses HSV and VSV (9). Although these examples demonstrate the principle of creating an improved retroviral vector for human gene therapy by pseudotyping, so far they have produced only vectors with extended tropism rather than with restricted specificities.

The logical and necessary extension of pseudotyping approaches, then, is to replace retroviral envelope genes with genes derived from nonviral sources. Although there are instances of nonviral glycoproteins being preferentially incorporated into retroviral particles, such as Thy-1 (10) and CD4 (11), actual infection of target cells, as opposed to specific binding, via display of such nonviral proteins has not been demonstrated,, and is likely to require either fusogenic sequences within the foreign protein itself or coexpression of fusogenic molecules on the viral envelope.

Engineering Env

Genetic manipulations whereby sequences conferring specific binding affinities are engineered into preexisting viral *env* genes represent a promising approach. In MoMLV the sequences that determine receptor specificity seem to be in the most distal of the two variable regions within the amino-terminal portion of the SU Env subunit, and replacement of the variable region of one strain with that of another can, for instance, change viral tropism from that of strain
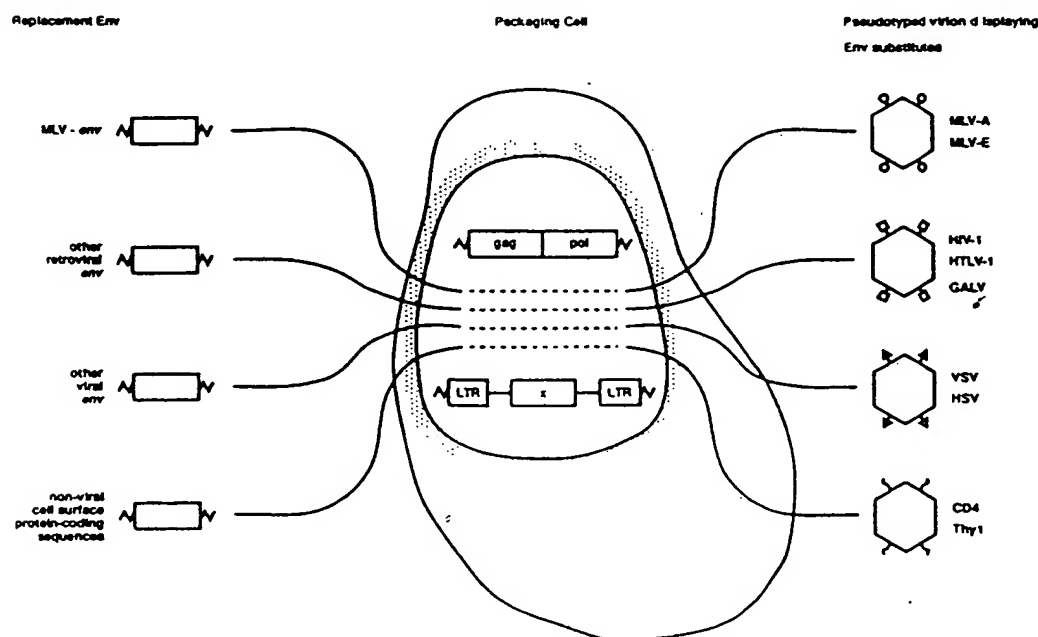
Figure 1. Generation of retroviral vectors with novel tropisms by construction of hybrid packaging lines. Transfection of a cell with genes (*gag-pol*, *env*) that encode viral *trans*-acting functions allows expression of all the structural components of the virion by that cell; these components can recognize and package the recombinant retroviral genome (shown here bounded by long terminal repeats (LTRs) and carrying a therapeutic gene x) Here we represent diagrammatically the various classes of retroviral pseudotypes that have been produced by providing various *env* genes in *trans*; this illustrates the principle of alteration of retroviral vector tropism by pseudotyping.

4070A to that of 10A1 (12). Engineering of murine retroviral Env proteins is being actively investigated (12-14) and is an important area of research. However, receptor recognition may involve complex interactions between the cellular ligand and different parts of the viral Env, and modification of viral tropism by direct replacement of receptor-binding sequences will not be straightforward. The function of Env proteins is not simply to adhere to host cells but also to participate in a sequence of events leading to membrane fusion. Excessive alteration of Env structure might therefore jeopardize the exposure of hydrophobic domains required for fusion and correct viral internalization. Nevertheless, a mammalian cell tropism has been conferred on an avian retrovirus by engineering integrin-binding sequences into Env. It was found that two of the variable regions of ALV Env could be manipulated by exchanging env sequences with those encoding a 16-amino acid RGD-containing peptide to produce Env proteins that were processed and incorporated into retroviral particles (15). Such hybrid envelopes could still efficiently mediate infection of avian cells through the ALV receptor, and could also infect and transfer neomycin resistance to mammalian (ALV-refractory) cells that expressed RGD-recognizing integrins. Infection was not efficient and required previous deglycosylation of the virus to expose RGD epitopes, but it is an important demonstration of the principle of targeting retroviral vectors by envelope modification.

In other studies, the RSV host range has been broadened to include human cells by packaging the genome with a chimeric Env that was a fusion of the RSV signal peptide and the influenza virus hemagglutinin (16). Chimeric Env was found to be incorporated into the virions as efficiently as wild-type RSV Env. It may be possible to use influenza hemagglutinins to direct retroviral vectors to subsets of cells exhibiting particular glycosylation phenotypes as the various influenza strains possess different hemagglutinins with different precise specificities. Another candidate protein for restriction of tropism is the B19 parvovirus surface protein, the surface receptor for which has recently been characterized (17) as the tetrasaccharide of globoside (blood group P antigen), which has a very limited tissue distribution. The B19 surface protein may be susceptible to fine-tuning of saccharide specificity by recombinant techniques or site-directed mutagenesis, similar to the influenza hemagglutinin (18).

The possibility of targeting retroviral vectors to particular glycosylation phenotypes may be of special interest for cancer therapy, as many transformed cells show altered glycosylation. Whether or not any aberrantly expressed glycans can mediate viral entry is another question; a recent report indicates that retroviruses targeted to cells via lectin cross-linking cannot infect the cells after binding (19), but this could be a function of the lectin or of structural alterations caused by cross-linking rather than a function of the glycan receptor.

The demonstrable ability (16) to alter RSV tropism from avian to human cells by manipulation of envelope structure could be of great interest for cancer therapy. This is because the vast number of target cells in malignant disease suggests that either the immune system must be recruited or that a replicating vector be used to target all the tumor cells, and RSV is a replicating vector par excellence. Besides its own genome, this virus is known to carry a cell-derived oncogene; replacement of this with a therapeutic cDNA would give a replication-competent gene therapy vector.

Encouraging results have been reported using a similar approach, in which a cDNA encoding an mAb fragment capable of hapten recognition was fused to the env gene of MoMLV (18). Coexpression of this gene with the normal envelope in an ecotropic packaging line resulted in infective viral particles that possessed the appropriate hapten-binding activity. It should be noted that the packaging line was expressing and required parental ecotropic Env as well as the chimeric protein, so it remains to be seen if infective retroviral particles can be assembled that contain only hapten-displaying Env (20). This approach has yet to be demonstrated using a hapten directed against a relevant human antigen capable of mediating virus internalization, and is still far from in vivo application.

Targeting by retrovirus-ligand conjugates

Hepatocytes possess a unique receptor that internalizes asialoglycoproteins. Conjugation of lactose to ecotropic viral particles allowed them to be recognized as asialoglycoproteins and broadened their host range to include human hepatoma cells (21). However, this approach is limited first to cells that express the asialoglycoprotein receptor, and second to proliferating cells (because retroviruses depend on host cell mitosis in order to integrate). As normal liver cells have a very low turnover rate, this technique is most likely to be of use for in vivo delivery to malignant liver disease of the hepatocyte lineage. Furthermore, because the vector was based on an ecotropic virus, its tropism in humans would be limited entirely to hepatocytes, greatly increasing its safety compared with broad affinity vectors such as those bearing the 4070A or GALV envelope proteins.

In a more indirect approach, it was found that ecotropic MoMLV vectors bound to human hepatoma cells after being cross-linked to the transferrin receptor by a series of antibodies; however, there was no subsequent proviral integration, suggesting either that the cross-linking antibodies were inhibiting membrane fusion or that the transferrin receptor cannot mediate appropriate viral internalization (22). A similar cross-linked mAb technique has been used to target ecotropic retroviral particles to human cells in vitro by means of the streptavidin-biotin reaction (23). This allowed ecotropic virus to bind to cells expressing human class I or II MHC antigens and to become internalized and integrated. An extension of this technique (19) showed that biotinylated EGF or insulin could substitute for the anticellular receptor antibody, and that EGF and insulin receptors could mediate internalization, leading to integration, of retroviral particles bearing streptavidin-conjugated antibodies. The possibility of targeting retroviral vectors by means other than murine antibodies, which suffer from numerous disadvantages in vivo, suggests that this approach may have potential although its in vivo applicability has yet to be demonstrated.

Adenoviral vectors

Adenoviruses are double-stranded DNA viruses in which the viral genomic DNA is contained in a virally encoded protein coat (capsid) rather than a phospholipid bilayer of host cell origin. The capsid consists of three major types of subunit: the hexon, which makes up the bulk of the coat; the penton base; and the penton fiber. The fiber is attached to the capsid via the penton base and projects outward; base and fiber together are known as the penton complex. During infection, the fiber mediates initial binding of the virus to an unidentified cellular receptor and the penton base subsequently mediates virus internalization via interactions with α_v-type integrins (24). Thus, the penton complex is respon-

ible for binding and internalization, and therefore for viral tropism at the level of cell recognition. Although adenoviral diseases are usually associated in vivo with respiratory epithelium or the GI tract, their cellular receptors seem to be widely distributed (25) Clearly then, as with retroviruses, the problem is to limit viral tropism to a particular subset of tissues. The adenoviral proteins responsible for attachment and internalization, respectively, have been well characterized, giving two points at which to manipulate tropism. The most promising approach is to restrict adenovirus infection at the cell-binding stage by replacing the carboxyl-terminus knob of the fiber with a ligand conferring a particular tropism, for instance, with an antibody hapten. One report (26) describes the restriction of adenovirus type 5 tropism by a different kind of fiber modification where intact virions were chemically modified so that their fiber carbohydrate groups were covalently linked to an asialoglycoprotein-polylysine conjugate. Such modified virus was found to have much decreased infectivity to asialoglycoprotein receptor-negative cells while retaining infectivity to receptor-positive cells. This approach would be equally applicable to targeting adenoviral vectors per se. It may also be possible to restrict infection by replacing the RGD-containing domain of the penton base with sequences having affinity for a ligand other than RGD-recognizing integrins.

Adenoviral vectors can also be targeted via the route of administration (27); targeting of a *lacZ*-expressing adenoviral vector to the kidney by renal artery or pyelic cavity infusion resulted in β-gal activity in various renal cells with no detectable expression in liver, lung, or bladder cells (27).

A possible advantage of refinement of vector targeting to the point of absolute specificity might be the ability to use replicating vectors for gene therapy. For cancer, development of a replicating adenoviral vector, perhaps carrying a cytokine or suicide gene, targeted to cancer cells at the level of cell binding (via fiber/base manipulations) and at the level of transcription (see next section) might allow transduction of the large number of malignant cells in a tumor deposit; cell death due to adenovirally induced lysis may even potentiate the field effect of cytokines. A safety feature of such a system would be that the immune system would be expected to eventually clear such therapeutic infections (as it does for wild-type infections); therefore this potential therapy only awaits adequate targeting strategies.

Liposome vectors

Most work on targeted liposomes has been designed to deliver cytotoxic drugs to cancer cells and has been reviewed recently (28). Expression of a cDNA in the target cells makes greater demands on the vector system in that it must not only target the appropriate cell type but also allow efficient delivery of undegraded DNA to the nucleus. For most targeted gene delivery purposes, conventional liposomes are limited because of their selective uptake by cells of the reticuloendothelial system (RES), in particular by macrophages resident in liver, spleen, and bone marrow, because of their limited extent of extravasation. Where macrophages themselves are the target, however, RES affinity is advantageous. In *L. donovani* leishmaniasis parasites not only multiply in the Kupffer cells of the liver, but are also resident in a vacuole to which lysosomes fuse, so that liposomes are passively targeted not only to the parasitized cell but also to the appropriate organelle, making liposome-mediated delivery of transcriptionally targeted antisense or suicide genes to these parasites a real possibility. It is also possible in a few cases to avoid much of the RES by the particular route of ap-

plication, particularly where the target tissue is found in a discrete anatomical compartment; e.g., nontargeted liposomes could be applied directly to the bladder for treatment of carcinoma or to the lung for treatment of cystic fibrosis or αAT deficiency Targeting by compartment has allowed confined transduction of discrete sections of arterial wall using both liposomal and retroviral vectors (29).

In most cases, however, in vivo use of liposomes requires first avoiding the RES, and second, display of appropriate tropic and fusogenic molecules (Fig. 2). Uptake by the RES can be considerably delayed, but not altogether avoided, by the use of "stealth" liposomes that display negatively charged moieties such as the ganglioside GMI and polyethylene glycol (PEG) (28). For most systemic purposes, the stealth formula is probably essential.

Liposomes bearing an immunoglobulin complement ("immunoliposomes") can exhibit tropisms conferred by the displayed antibody. Hence, coupling to liposomes of an antibody against glioma cells increased the efficiency of gene delivery to these cells in culture by about sevenfold (30). Just as mAbs may be conjugated to liposomes to confer targeting capability, so may other ligands such as growth factors and hormones. Coupling of transferrin to liposomes followed by i.v. injection in a rabbit model resulted in significantly greater localization to bone marrow erythroblasts (31), and incorporation of surfactant protein A into liposomes increased the uptake of the liposome cargo by alveolar type II cells (32). However, it is not sufficient merely to confer upon the vector a particular binding ability; the particle must bind to a ligand that also allows fusion of liposome and cell membranes. Such considerations of appropriate internalization of vector cargo are especially important for gene delivery vectors, where the DNA must not only reach the appropriate cell type but also must reach the nucleus in undegraded form.

Conjugating virions to liposomes or incorporating viral surface glycoproteins into liposomes might create a vector that has the efficient cell attachment and entry mechanisms of a virus but not the safety drawbacks; much work has been done in this area with Sendai virus in particular (33). Another system used liposomes that displayed only the fusogenic protein of Sendai virus (F-protein) and not the cell-binding protein (hemagglutinin) (34). However, although
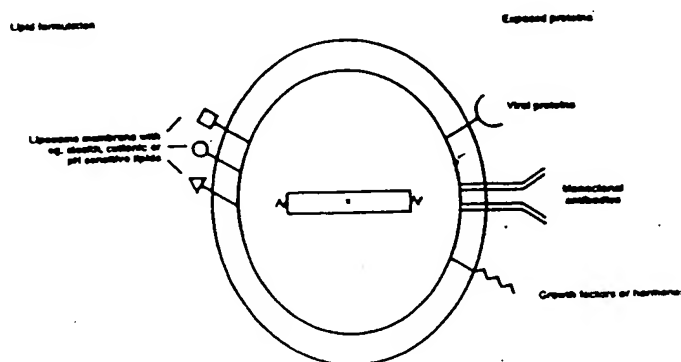
Figure 2. Modification of lipid membranes to produce targeted liposomes. Targeting of liposomes requires first abrogation of their RES affinity, and second, provision with exposed ligands having the required targeting capacity. Inclusion of ganglioside glycolipids into the lipid formulation can allow RES evasion; other lipid formulations include cationic lipids to allow promiscuous membrane binding and hence lysosome escape, and pH-sensitive lipids, which allow lysosome escape without the broad affinity conferred by cationic lipids. Various types of ligand can be inserted into the lipid membrane for provision of particular tropisms (see text for details).

such approaches can make liposomes up to 10-fold more efficient than lipofection at gene delivery (33), in terms of targeting all it can do is confer upon the liposome the tropism of the virus, and there are very few native viral receptors that exhibit a narrow and precise cell type specificity. Nevertheless, a promising system (35) is currently being developed in which respiratory epithelium is targeted by means of the surface proteins of respiratory syncytial virus (ReSV), which is responsible for infections of the lower respiratory tract. Liposome-type envelopes were constructed that displayed both the attachment and fusion proteins of ReSV, and these have been shown to enter all cells of a cultured respiratory epithelial cell line within 1 h (35)

Cationic liposomes such as the commercially produced lipofectin can efficiently avoid the lysosomal pathway because the particular lipid composition allows direct fusion of liposome and cell membranes. These particles are therefore much more efficient than conventional liposomes, and for in vitro transduction have largely replaced them. Cationic liposomes have also been used for in vivo approaches and even clinical trials; however, there seem to be no data on the extent to which these liposomes can avoid the RES, and indeed the cationic surface would seem to be incompatible with the negative charges characteristic of the stealth formulation. One report suggests that the cationic liposome has as much affinity for other cell types as for the RES after i.v. injection (36). Administration of liposomes carrying SV40-CAT resulted in widespread expression of the marker gene for up to 9 wk, albeit mainly in tissues generally associated with the RES such as spleen, liver, lymph nodes, and bone marrow as well as in vascular endothelium. CAT expression was also observed in tumor cells in this experiment, probably as a

consequence of leaky tumor vasculature. It may eventually be possible to combine the efficient lysosomal avoidance of cationic liposomes with a specific targeting capacity although the problem is likely to be that the generally fusogenic nature of cationic liposomes may preclude any precisely restricted targeting

Molecular conjugate vectors

Targeting of plasmid DNA may be achieved by coupling the DNA to a ligand with a demonstrated cell or tissue affinity. This is usually brought about by covalently linking a polycation such as polylysine to the ligand; the polycation can then bind to and condense plasmid DNA via electrostatic interactions, leaving the ligand exposed on the surface of the conjugate (37). The ligands chosen must be efficiently endocytosed in the target cells so that DNA is efficiently internalized. One of the first receptors to be used in this way was the asialoglycoprotein receptor, whose expression is limited to hepatocytes; this receptor binds glycoproteins with terminal galactose residues for removal from the circulation; asialoorosomucoid (ASOR) is a major natural ligand for this receptor. BSA has been given specificity for the ASOR receptor by artificial galactosylation, and has been used to target CAT and human factor IX cDNAs (38) to hepatoma cells in vitro and to liver but not other tissues in vivo. Other ligands that have been used in similar conjugates include insulin (39), EGF (40), lectins (41), and transferrin (37). A major drawback of classical molecular conjugate vectors is that internalization depends on receptor-mediated endocytosis, a process that directs the receptor complex to lysosomes where it is degraded; only a small fraction of introduced
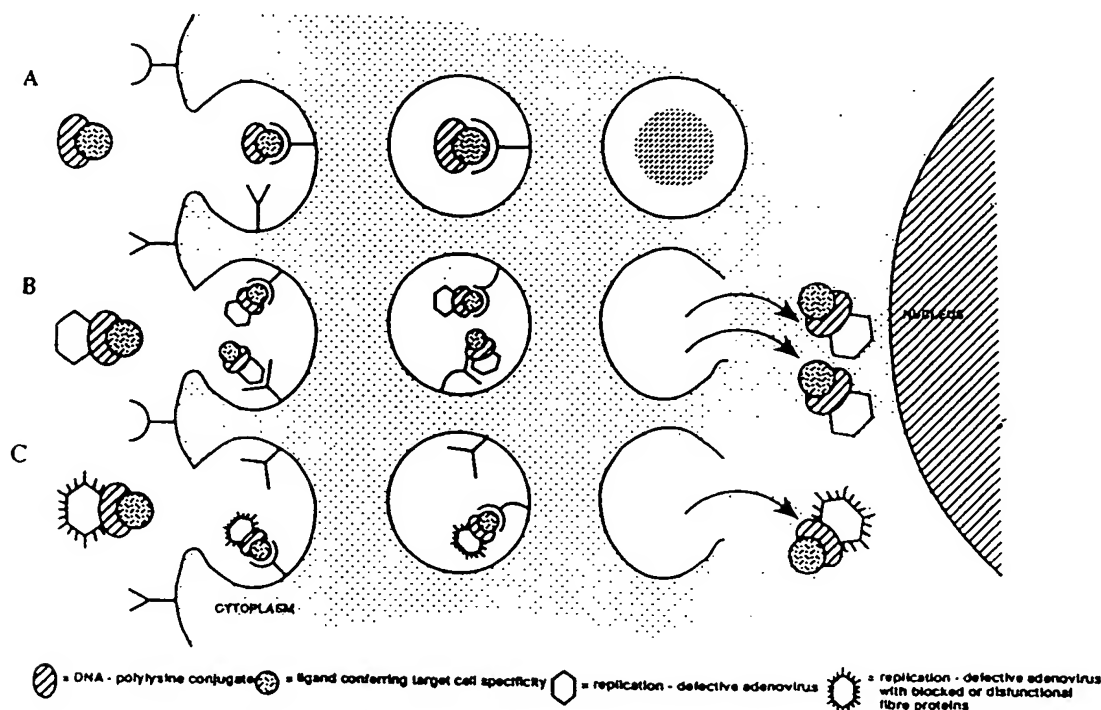
Figure 3. Targeting of plasmid DNA by molecular conjugate vectors. Conjugation of plasmid DNA to a particular ligand can confer a particular targeting capacity, but results in a vector of very low efficiency because most receptor-mediated endocytosis directs such conjugates to lysosomes where the great majority of vector DNA is degraded (route A). By complexing an adenovirus coat to the conjugate, a highly efficient vector is created by virtue of the ability of adenovirus proteins to disrupt the endosome before vector degradation (route B); however, this abrogates any targeting capacity conferred by the ligand, as the complex can enter cells either via the ligand receptor or via the virtually ubiquitous adenoviral receptor. To truly target such complexes it will be necessary to use modified adenoviral coats that retain the lysosomal escape mechanism but cannot interact with the adenoviral receptor (route C).

DNA escapes this pathway and enters the nucleus. leading to low efficiency of transduction

A new generation of molecular conjugate vectors has been produced that has the capacity to escape the degradative lysosomal pathway by utilizing features of the adenovirus capsid (Fig. 3). Adenovirus disrupts endosomes during cell entry as a consequence of a conformational change in the capsid proteins, resulting in membrane breakdown, triggered by a drop in pH. Hence, molecular conjugate vectors delivered DNA to cells with greatly increased efficiency when transfection was done in the presence of adenovirus. However, this effect relies on both virus and vector being present in the same endosome. To improve efficiency, the adenovirus has been coupled directly to the molecular conjugate (37). However, adenovirus receptors are virtually ubiquitous and so the coupling of an adenovirus receptor to a targeted molecular conjugate would be expected to partially or completely abrogate any preferential tropism conferred by the ligand. Blocking the interaction of fiber with adenovirus receptor by mAb to the fiber resulted (42) in a vector that was both targeted to a specific subset of cells and able to escape the lysosomal pathway. A more satisfactory approach would be to create recombinant adenoviral vectors that display dysfunctional fiber proteins in order to bypass the antibody-coating step.

Few in vivo experiments have been attempted using adenovirus-molecular conjugate complexes, and in fact it is unlikely that such vectors will be routinely applicable to in vivo work, although they are likely to be of use for ex vivo strategies (43). This is a consequence first of the size of the complex (transferrin-polycation conjugates are approximately 100 nm in diameter (44); complexed with AdV they would be even larger), which will prohibit extensive extravasation or tissue penetration, and second, of the likelihood of direct immunogenicity of the AdV proteins (45).

TARGETING OF GENE THERAPY VECTORS AT THE GENETIC LEVEL

Transcriptional targeting

Therapeutic cDNAs may be limited in expression to a particular subset of cells by placing them under the control of regulatory elements that possess binding sites for tissue-restricted positive or negative trans-acting factors (Fig. 4). Correctly regulated expression may require, in addition to 5' promoter sequences, distant elements either 5' or 3' to the coding region; these elements act together with the promoter and allow tissue-specific expression at appropriate levels independent of position of integration. Such locus control regions (LCRs) have been identified for a number of genes. LCRs would be of much use for gene augmentation but the transfer of such large sections of DNA to target cells will be problematic, particularly in vivo, and in fact for the foreseeable future may be confined to ex vivo strategies. Where a monogenic defect results in pathology in more than one tissue, the most pragmatic approach to appropriately limit the expression of therapeutic cDNA is to use the cellular promoter/enhancer elements native to the defective gene. Furthermore, the use of cellular rather than viral promoters reduces the chance of loss of cDNA expression due to inactivation of viral sequences by methylation or other mechanisms (46). Thus, cellular promoters may confer benefits both of long-term expression and of tissue-restricted expression, and where vector-targeting at the cell-binding level has not been achieved it may represent the only way of limiting expression of exogenous cDNA.
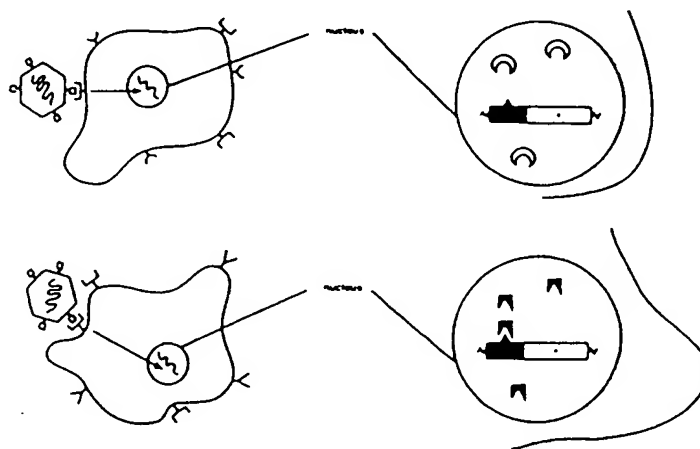
Figure 4. Tissue-restricted transcription. A promiscuously binding vector can be targeted at the transcriptional level if the therapeutic gene (x) is controlled by 5' regulatory elements (shown here as a shaded region upstream of x) active only in the presence of tissue-specific nuclear transcription factors; thus expression of x occurs only in the target cells.

Tissue-specific cellular regulatory elements have great potential for development of safe, targeted vectors for gene therapy. For example, the creatine kinase promoter has been used in a plasmid vector to restrict dystrophin cDNA expression to skeletal and cardiac muscle, and in the mdx mouse model of Duchenne muscular dystrophy, mice transgenic for this promoter-cDNA construct were found to exhibit correction of dystrophic symptoms (47). A potential approach to the treatment of B cell lymphoma involves expression of suicide genes transcriptionally regulated by promoter/enhancers from the Ig heavy chain or the x light chain genes; expression plasmids containing the diphtheria toxin A (DT-A) gene controlled by these regulatory elements mediated significant expression of DT-A in B lymphoid cells but not in HeLa cells or fibroblasts (48).

Endothelial cells are attractive recipients for gene transfer therapies not only for obvious purposes such as targeting of tumor vasculature or therapy of cardiovascular disease, but also for the systemic secretion of therapeutic factors. An endothelial cell-specific regulatory region has recently been characterized (49) as 500 bp of 5' sequences, associated with the gene for von Willebrand's factor, acting in conjunction with an essential region in the first intron. This promoter could be particularly useful when driving a suicide gene in a retroviral vector as it would then be targeted to dividing endothelial cells, i.e., almost exclusively tumor vasculature.

Tissue-specific cellular promoters frequently retain their specificity in the context of a retroviral vector (50); however, this is not always the case, and the design of the retroviral vector may have significant effects on tissue specificity due to promoter interference (51). Tissue-specific promoters have also been shown to appropriately restrict cDNA expression in the context of recombinant adenoviruses, e.g., the rat albumin promoter maintained its hepatoma cell specificity in vitro (52), albeit at low levels.

Antiviral therapy using transcriptional targeting

Transcriptional targeting may be of particular use in the therapy of particular kinds of viral infection. In cases where the viral life cycle depends on self-encoded autoregulatory

proteins, vectors can be made in which therapeutic cDNAs are transcriptionally regulated by these same viral proteins. Transcription of the therapeutic cDNA is therefore limited to cells that are infected by the virus, and thus such an approach could be either prophylactic or curative. This strategy has been applied to experimental HIV therapies. One recent report (53) described the construction of a recombinant retrovirus containing HSV-TK driven by the HIV-2 LTR-TAR; cells expressing this construct became susceptible to ganciclovir after infection by HIV-2 in vitro.

Targeting proliferating cells

Murine C-type retroviral vectors can combine the ability to express cDNA from an internal tissue-specific promoter with an innate tropism for proliferating tissue. Therefore, they have great potential as vectors for the gene therapy of cancer, because restricted cDNA expression is of particular importance in strategies that involve delivery of cytokine or suicide genes and malignancies are often distinguished by rapid division in a relatively quiescent background. Indeed, in a very few cases the retroviral requirement for cell division may be sufficient in itself to target the therapy (Fig. 5); where tumors arise in the CNS their high rate of proliferation in the context of a completely postmitotic tissue, in an anatomical compartment that is separated from the rest of the body, allows efficient targeting with retroviral vectors (54). As an additional targeting feature for malignancies of the CNS, the glial-specific promoter region of the mouse myelin basic protein gene has been used to drive HSV-TK in a retroviral vector (55); this approach could allow long-term administration of producer cells at the primary site or systemic vector application to treat metastatic deposits as collateral infection of nonglial cells would not result in expression of the suicide gene.

Retroviral vectors would also be useful in targeting liver malignancies, as the liver is also slowly proliferative under normal circumstances. Tissue-specific promoters would be essential for such strategies, because unlike the CNS, the liver is not efficiently insulated from the rest of the body. Amphotropic retroviral vectors have been constructed carrying HSV-TK cDNA driven either by the albumin or the a-fetoprotein promoters (56). The albumin promoter was active only in cells of the liver lineage; the a-fetoprotein promoter conferred an extra level of targeting in that it was hepatoma-specific as opposed to hepatocyte-specific (a-fetoprotein is normally expressed only in fetal tissues).

The 5' region of the tyrosinase gene has also been used to restrict expression of therapeutic cDNAs to melanocytes and melanoma cells both in vitro and in vivo by means of retroviral vectors (51, 57). This kind of transcriptional targeting may be useful in VDEPT approaches for melanoma because normal melanocytes are dispersed and of low density in body tissues, and their ablation is likely to be minimally pathological. Even better would be the usurpation of tumor-specific transcriptional regulation by using promoter sequences from genes whose overexpression is limited to transformed tissue. One such candidate is the oncogene ERBB2, which is overexpressed in a variety of tumors. The ERBB2 promoter sequences have been used to drive cytosine deaminase cDNA in a retroviral vector (58); this strategy conferred sensitivity to ERBB2-overproducing cells but not to control cells, and represents a potentially widely applicable method of tumor-preferential transcriptional targeting. The a-fetoprotein promoter is in effect completely tumor-specific, but is applicable only to malignancies of the liver.

Exploitation of natural viral tropisms

An obvious approach to the precise targeting of tissues is to make vectors from viruses that have preferential patterns of transcription in target tissues, such as HSV vectors for nervous tissue. However, careful dissection of the genomes of these viruses will be necessary to separate pathogenic sequences from those that confer transcriptional specificity; in most cases it will be preferable to use cellular promoters in the vector of choice, especially as the range of transcriptionally targeted viral genomes is not great.

There may be one remarkable exception to the general requirement for cellular promoters rather than viral promoters in gene therapy, namely, the use of autonomous parvoviral sequences for targeting transformed cells (see ref 59 for review). These viruses preferentially kill transformed cells (60), and coinjection of mouse minute virus (MVM) and Ehrlich ascites tumor cells into the peritoneal cavities of mice inhibited tumor formation by up to 90%. Furthermore, mice that had survived one such coinjection were resistant to a second tumor challenge 5–6 wk later. The precise basis of parvovirus oncotropism is not understood but may be related to an effect of the transformed cell environment on the production or activity of parvovirus autoregulatory proteins. The parvovirus promoter that is preferentially transactivated in certain transformed cells is clearly a candidate to control transcription of suicide or cytokine genes in parvovirus vectors for cancer therapies. Recombinant parvovirus vectors have been made and shown to both transfer exogenous cDNA expression to recipient cells and retain their oncotropism in vitro (61) for human and murine cells. Recombinant parvoviruses may therefore represent one of the most promising approaches to cancer therapies for the future.
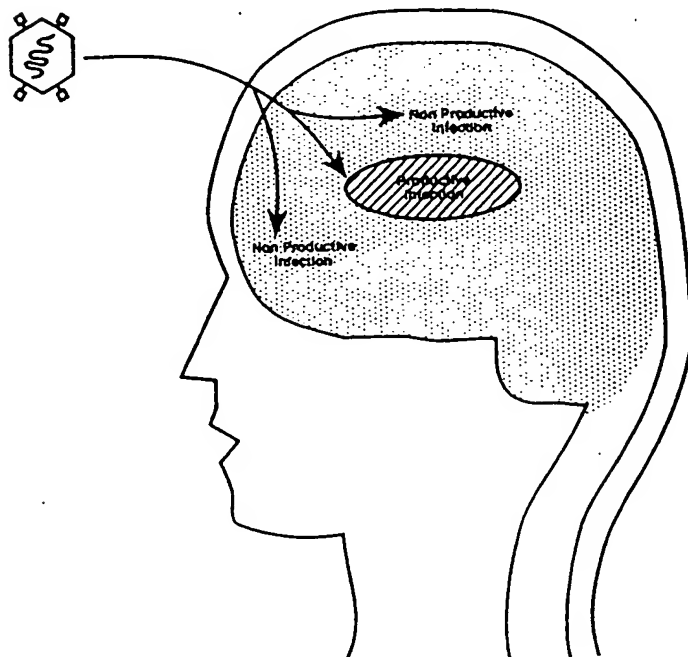
Figure 5. Targeting proliferating cells. Retroviral vectors require cell division for integration and gene expression; therefore where a tumor arises in a completely postmitotic background, such as the CNS, the proliferation of the malignant tissue may be sufficient in itself to allow efficiently targeted delivery of suicide genes via recombinant retroviruses. Actively replicating (tumor) cells are represented by diagonal lines; quiescent neuron tissue is represented by dots.

Targeted integration: site-specific recombination

Nonintegrating vectors are adequate for transient expression of cDNA. Where the object is a "one-shot" treatment for cure of a genetic disorder, it is necessary to use either an integrating vector or a stably replicating extrachromosomal element. For the future, sequences containing mammalian origins of replication or even entire mammalian artificial chromosomes (62) could have great potential especially for ex vivo approaches. Similarly, vectors based on the Epstein-Barr virus, which is stably maintained episomally as a plasmid in human cells, may one day be suitable for clinical use.

The ideal approach would be to target the exogenous DNA to the mutant gene, i.e., gene replacement rather than gene augmentation. Such gene targeting approaches may be of use for ex vivo strategies to stably transduce cells with less likelihood of simultaneous transformation (63). Such in vitro homologous recombination may be useful in inactivating genes responsible for MHC class I expression in myoblasts to create a universal carrier cell that can be transplanted regardless of the recipient HLA type (63). This approach is applicable to any ex vivo strategy that requires implantation of viable transduced but otherwise unchanged cells. The technology required to accomplish this at levels of efficiency relevant to in vivo gene transfer does not yet exist and so integrating gene therapy vectors at present can offer only gene augmentation.

Nontargeted integration could be hazardous if completely random, not only by turning on downstream oncogenes via promoter readthrough but also by direct disruption of genes, and this is the main source of concern with regard to the use of retroviral vectors in humans. Vectors with the capacity for site-specific integration would overcome these problems. Adeno-associated virus is a defective parvovirus that potentially is widely applicable in gene transfer strategies because it is tropic for many cell types, nonpathogenic in humans (in the absence of helper virus the AAV genome does not replicate but integrates into the genome and assumes a state of latency), and can be manipulated to derive recombinant genomes capable of vectoring exogenous DNA (64). Although these vectors can package only up to 4.5 kb as compared with the retrovirus limit of approximately 7 kb, they are said to have one major advantage over other integrating vectors, namely, a propensity (which is far from total) for apparently harmless integration into a region of human chromosome 19 known as AAVS1 (see review, ref 65). Where such specific integration occurs, it is almost certainly mediated by virally encoded proteins with affinity both for the target site and for the virus genome (66). Although integrated viral sequences remain dormant until superinfection by AdV/HSV, exogenous cDNAs driven by internal promoters can still be active (furthermore, the transcriptional inactivity of the viral ITR means that there will be no promoter interference leading to, for example, loss of tissue specificity of exogenous promoter, and less chance of insertional mutagenesis for the same reason). Thus AAV vectors have been shown to confer neomycin resistance and in some cases to integrate with site specificity (64). This study also showed that AAV vectors preserved their site specificity after transfection in plasmid form; the use of a transfectable plasmid rather than a viral vector might overcome the packaging limitations of AAV vectors (64). It must be said, however, that some groups report that recombinant AAV vectors show site specificity in only a relatively minor proportion of the total number of integration events. There have been several attempts to explore the therapeutic potential of AAV vectors, e.g., the delivery of cDNA for the correction of the cystic fibrosis defects (67).

There may be other vector systems also capable of site-specific integration. Eukaryotic genomes harbor large numbers of endogenous transposable elements of various types (68), i.e., autonomously replicating units that can insert themselves into the host genome. Some of these elements, known as LTR retrotransposons, are very similar to retroviruses both in replication cycle and in organization, being bound by LTRs and possessing coding regions with homology to retroviral gag-pol genes. The replicative cycle of LTR retrotransposons exactly parallels that of the retroviruses except that there is no envelope stage, thus, cytoplasmic virus-like particles (69) are formed containing reverse transcriptase, the RNA form of the retrotransposon, and cellular tRNA primers for reverse transcription. Such elements include copia, yeast Ty, and the intracisternal A particle of mice; clearly they have great potential as vectors of improved safety as their use with retroviral packaging lines would be less likely to result in helper virus production through homologous recombination. Indeed a mouse retrotransposon VL30 has already been made into a gene transfer vector (70), which can be produced in a standard retroviral packaging line. Endogenous retrotransposons a priori would be expected, through coevolution with the host genome, to display a degree of site specificity of integration as continuous random retrotransposition would be deleterious to the cell. Yeast retrotransposons offer the best examples of site-specific retrotransposons, and moreover, their site of integration appears to be benign. Two of the five *Saccharomyces cerevisiae* retrotransposons, Ty1 and Ty3, exhibit unambiguous site specificity of integration (71). Ty3 elements integrate into sites upstream of genes transcribed by RNA pol III, frequently within 1–4 nucleotides of the start site of transcription. It has been suggested that this sequence-independent site specificity is brought about by interaction of the retrotransposon with elements involved in RNA pol III-mediated transcription, e.g., TFIIIB (71). Similarly, Ty1 preferentially integrates upstream of tRNA genes (71) 57% of insertions occurring within 400 bp of a tRNA gene. A consequence of this specificity is that yeast genes are only rarely interrupted by Ty1 insertions as regions upstream of yeast tRNA genes rarely contain open reading frames (71). The great similarity of LTR retrotransposons to retroviruses allows them to be made into vectors with conventional retrovirus packaging lines (70); possibly the development of a packaging line that provides retrotransposon rather than retroviral gag-pol in trans will allow the production of vectors with integrational site specificity.

SUMMARY AND PERSPECTIVES

Of the gene therapy protocols that have so far entered clinical trials, targeting of the appropriate vectors has been achieved largely only by indirect means. Thus, several such trials (for example, for treatment of ADA deficiency, HIV infection, or cancer) have used specific cell populations that have been removed from the patient and infected in vitro by nontargeted amphotropic retroviruses before being returned in vivo. Further levels of targeting have been achieved in some cases by careful choice of the patient's cells; for instance, ex vivo transduction of tumor infiltrating lymphocytes with potentially tumoricidal genes has been proposed as a means of delivering their products to tumor deposits at much higher concentrations than would otherwise be possible.

In contrast to ex vivo manipulation of target cells where the vector requires very little, if any, intrinsic targeting capability, there are an increasing number of protocols in which
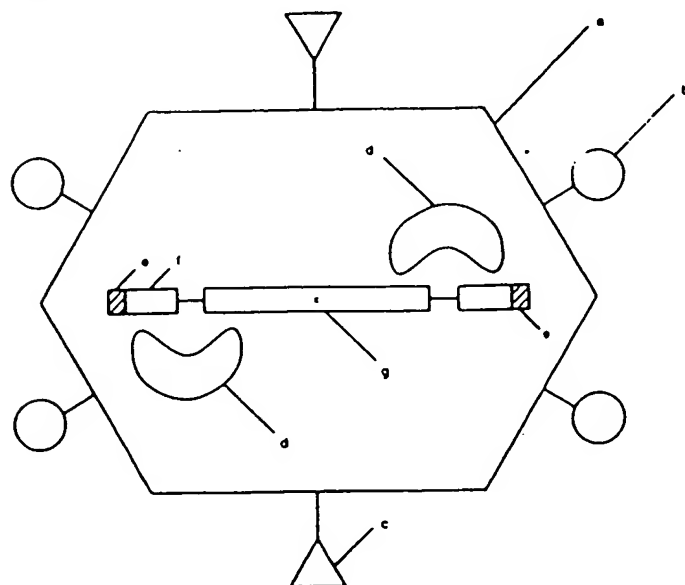
Nonetheless, the imagination and the technology is currently available to allow us to hope that vectors will eventually be constructed that can include both efficiency and specificity. In particular, it does not seem unrealistic to suppose that the gene therapy vectors of the future will not be based exclusively on any single virus or physical vector system alone but will be synthetic, custom-designed vehicles (Fig. 6) into which specific targeting features can be included depending on the particular clinical requirements of the target disease and tissue. $\boxed{FJ}$

We would like to thank Professor Bob Williamson for influential discussions.

Figure 6. A theoretical composite vector Some features that might be incorporated in an ideal synthetic vector include a stable, nonimmunogenic envelope, probably lipid (*a*); exposed ligands to confer a particular affinity on the vector (*b*); moieties that encourage fusion between vector and target cell membranes (*c*); proteins to allow directed integration of vector DNA, e.g., site-specific recombinases (*d*); sequences to enable homologous recombination between vector DNA and particular loci of the target genome (*e*); tissue-specific promoter regions to allow restricted expression of the therapeutic gene (*f*); and the therapeutic cDNA (*g*).

recombinant genes are delivered directly to patients in vivo (such as for the treatment of cystic fibrosis and cancer). Once again, targeting at the level of the vector has not yet been particularly well developed; hence, liposome or viral-mediated delivery of the CFTR gene to airway epithelial cells of CF patients has relied largely on the localized delivery of the vectors directly to the affected tissues, and on the fact that there is good evidence that inadvertant expression of the CFTR gene in cells other than the target epithelial cells may have few adverse effects. Localized delivery has also been used in the treatment of brain tumor deposits, using stereotactic injection of retroviral producer cells, but with the added sophistication that the retroviruses would be expected to infect only the actively dividing tumor cells and not the surrounding neural tissue.

However, for the long-term success as well as the widespread applicability of human gene therapy, there will have to be advances in the ability with which clinicians can confidently administer recombinant vectors for the treatment of genetic disease directly to affected tissues in vivo. For this to occur, many targeting strategies outlined in this review, which are currently only at the experimental level, will have to be translated into components of safe and highly efficient delivery systems. Vectors have already been developed that incorporate transcriptional specificity for a certain tissue type; however, the development of surface targeting has been more problematic in most cases. The biggest challenge for the next 5 years will be to combine targeting with efficiency in the production of the vector systems of the future. So far, attainment of one usually compromises the other; for example, we have constructed retroviral vectors targeted at the level of transcription to melanoma cells but these viruses are generally of lower titer than their nontargeted counterparts.

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EXHIBIT 10

REVIEW

Components of Vectors for Gene Transfer and Expression in Mammalian Cells

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Progress in diverse scientific fields has been realized partly by the continued refinement of mammalian gene expression vectors. A growing understanding of biological processes now allows the design of vector components to meet specific objectives. Thus, gene expression in a tissue-selective or ubiquitous manner may be accomplished by selecting appropriate promoter/enhancer elements; stabilization of labile mRNAs may be effected through removal of 3' untranslated regions or fusion to heterologous stabilizing sequences; protein targeting to selected tissues or different organelles is carried out using specific signal sequences; fusion moieties effect the detection, enhanced yield, surface expression, prolongation of half-life, and facile purification of recombinant proteins; and careful tailoring of the codon content of heterologous genes enhances protein production from poorly translated transcripts. The use of viral as well as nonviral genetic elements in vectors allows the stable replication of episomal elements without the need for chromosomal integration. The development of baculovirus vectors for both transient and stable gene expression in mammalian cells has expanded the utility of such vectors for a broad range of cell types. Internal ribosome entry sites are now widely used in many applications that require coexpression of different genes. Progress in gene targeting techniques is likely to transform gene expression and amplification in mammalian cells into a considerably less labor-intensive operation. Future progress in the elucidation of eukaryotic protein degradation pathways holds promise for developing methods to minimize proteolysis of specific recombinant proteins in mammalian cells and tissues. © 1999 Academic Press

In recent years progress in the design, sophistication, and availability of vectors for gene expression in mammalian cells has been phenomenal. Vectors have many applications, including the study of gene regulation, DNA sequencing, molecular cloning, protein pro-

duction, antigen expression for vaccination, and gene therapy. There is a large number of vectors available; for example, Vector Database on the Web (Table 1) lists more than 2600 vectors. In spite of the plethora of available vectors, however, robust protein production in mammalian cells is not necessarily a routine matter. Efficient expression of genes in mammalian cells depends on many factors, including both transcriptional and translational control elements, RNA processing, gene copy number, mRNA stability, the chromosomal site of gene integration, potential toxicity of recombinant proteins to the host cell, as well as the genetic properties of the host. Gene transfer into mammalian cells may be effected either by infection with virus that carries the recombinant gene of interest or by direct transfer of plasmid DNA. Due to space limitations, the emphasis here is on nonviral vectors for high-level protein production. There is extensive literature on mammalian vectors of viral origin with applications in protein production, gene therapy, and vaccine development. The reader is referred to recent reviews (1–7) and to Table 2 for selected references on viral-based vectors. In addition, only brief coverage of inducible vector systems is provided here, as several excellent reviews have covered this topic in detail (8–13).

The choice of an expression system for production of recombinant proteins depends on many factors, including cell growth characteristics, expression levels, intracellular and extracellular expression, posttranslational modifications and biological activity of the protein of interest, as well as regulatory issues and economic considerations in the production of therapeutic proteins (14–16). Key advantages of mammalian cells over other expression systems are the ability to carry out proper protein folding, complex *N*-linked glycosylation and authentic *O*-linked glycosylation, as well as a broad spectrum of posttranslational modifications (14). The essential elements of mammalian expression vectors (Table 3 and Fig. 1) include (1) a constitutive or inducible promoter capable of robust transcriptional activity; (2) optimized mRNA processing and transla-

tional signals that include a Kozak sequence, translation termination codon, mRNA cleavage and polyadenylation signals, as well as mRNA splicing signals; (3) a transcription terminator; (4) selection markers (Table 4) for the preparation of stable cell lines and for gene amplification; and (5) prokaryotic origin of replication and selection markers for vector propagation in bacteria. The inclusion of the SV40 origin of replication facilitates transient gene expression in COS cells. Other genetic elements for specific applications include fusion moieties, protease cleavage sites, sequences for gene or protein targeting, and IRES elements for the construction of polycistronic vectors (Fig. 1D).

TABLE 1
Web Databases for Gene Expression Vectors

Vector Database
http://vectordb.atcg.com
  VectorDB contains information on more than 2600 vectors, including phage, plasmid, phagemid, phasmid, cosmid, viral, and YAC vectors. The database has a search engine and contains annotation and sequence information for many of the vectors. In addition, vectors which are also in GenBank have direct links to that database.
Gene Transfer Vector Core
http://www.uiowa.edu/~gene
  This site is from the University of Iowa College of Medicine. The core produces viral and nonviral vectors in quantities necessary for gene transfer in research experiments or preclinical studies. Core staff work closely with investigators to plan and develop gene transfer vectors to fit individual project requirements. Customized virus constructions are available on a fee for service basis.
Gene Therapy Vectors
http://www.wiley.co.uk/genetherapy/vectors.html
  This site deals with all aspects of gene therapy, including an overview of gene delivery systems and illustrations of the most widely used gene transfer vectors. The site also provides comprehensive summaries of clinical trials in gene therapy worldwide and has links to information from meetings, published material, regulatory agencies, and related databases.
Course BS335: Virology
http://www-micro.msb.le.ac.uk/335/BS335.html
  This course on the main principles of virology is organized by Alan Cann from the University of Leicester. The site includes a detailed section on viral vectors for gene transfer and therapy organized by David Peel.
National Gene Vector Laboratories (NGVL)
http://www.iupui.edu/~iucc/ngvl
  The NGVL are funded by the National Institutes of Health and are composed of a group of academic laboratories whose goal is to provide eligible investigators with clinical grade vectors for gene therapy. The home page includes the following links: *NGVL at Indiana University* produces retroviral and AAV vectors. *NGVL at the University of Michigan* produces nonviral vectors. *NGVL at the University of Pennsylvania* produces adenoviral vectors.
Sindbis Virus Gene Expression Vectors
http://www.microbiology.wustl.edu/Sindbis/sinVectors
  This site from Washington University in St. Louis provides a detailed description of the molecular biology of Sindbis virus, including a bibliography and types of Sindbis virus gene expression vectors.

TABLE 2
Viral-Based Vectors for Gene Transfer and Expression in Mammalian Cells

Viral-based vector	Reference No.
DNA viruses	
Cytomegalovirus	148
Herpes simplex virus	149
Epstein-Barr virus	150, 151
Simian virus 40	20, 21
Bovine papillomavirus	49
Adeno-associated virus	152
Adenovirus	153, 154
Vaccinia virus	155, 156
Baculovirus	157
RNA viruses	
Semliki Forest virus*	158, 159
Sindbis virus	160, 161
Poliovirus	162, 163
Rabies virus	164
Influenza virus	165, 166
SV5	167
Respiratory syncytial virus	168
Venezuelan equine encephalitis virus	169, 170
Kunjin virus	171
Sendai virus	172
Vesicular stomatitis virus	173
Retroviruses	2, 174, 175
Chimeric viral vectors	
Adenovirus-Sindbis virus	176
Adenovirus-adeno-associated virus	177

* A DNA-based self-amplifying SFV vector has been developed (178).

1. TRANSIENT GENE EXPRESSION

Transient gene expression is a convenient method for the rapid production of small quantities of protein for initial characterization. In addition, the method lends itself to the rapid testing of vector functionality as well as optimization of different combinations of promoters and other elements in expression vectors. Thus, once the appropriate vector has been constructed, results from transient expression assays can be obtained in 2 to 3 days and allow one to proceed with confidence to the more time-consuming task of preparing permanent stable cell lines for protein production on a larger scale. There are several cell types used for transient gene expression, including COS, baby hamster kidney (BHK)[1], and human embryonic kidney (HEK)-293 cells, as well as genetically modified HEK-293 cells (reviewed in 17). The most widely used transient expression system utilizes COS cells (reviewed in

[1] Abbreviations used: BHK, baby hamster kidney; HEK, human embryonic kidney; S/MAR, scaffold/matrix attached region; CHO, Chinese hamster ovary; Cre, cyclization recombination; MDCK, Madin–Darby canine kidney cells; MMP13, metalloproteinase 13; IPTG, isopropyl β-D-thiogalactopyranoside; UTR, untranslated region; OTC, ornithine transcarbamylase; GFP, green fluorescent protein; IRES, internal ribosome entry site.

18,19). COS cell lines were generated by the transfection of African green monkey kidney CV1 cells with an origin-defective SV40 (20,21). COS cells express the SV40 T antigen, which allows replication of plasmids containing the SV40 origin of replication. This host/vector system facilitates high-level plasmid amplification and protein production, followed by lysis of the cells 3 to 4 days from the time of transfection. COS cells have been used for the transient expression of numerous genes, including the production of a wide range of monoclonal antibodies (19). It is also possible to use COS cells on a large scale for the production of milligram quantities of protein, thus obviating the need for multiple transfections (17).

2. STABLE GENE EXPRESSION

In contrast to transient gene expression, preparation of stable cell lines that "permanently" express the gene of interest depends on the stable integration of plasmid into the host chromosome. It is also possible, however, to generate stable cell lines that harbor vectors extrachromosomally. For example, vectors that carry the Epstein-Barr virus nuclear antigen (EBNA-1) and the origin of replication (oriP) (Table 3) can be maintained episomally in primate and canine cell lines but not in rodent cell lines (22). Recently, an episomal replicating vector has been described that does not express any viral proteins, thus avoiding cell transformation (23). The vector contains the SV40 origin of replication and the scaffold/matrix attached region (S/MAR) from the human interferon-β gene. S/MARs are DNA sequences associated with chromosomal origins of bidirectional replication. The vector was shown to replicate at very low copy numbers (below 20) in Chinese hamster ovary (CHO) cells and was stably maintained without selection for more than 100 generations (23).

The choice of host cell may have a significant impact on gene expression levels. For example, myeloma cells have been mainly used for high-level production of monoclonal antibodies (19). However, amplifiable expression systems using CHO cells have been widely used for the successful production of proteins of therapeutic interest. There are many genes that confer drug resistance upon amplification (24); however, the two most widely used amplification systems rely on the dihydrofolate reductase and glutamine synthetase genes. Thus, by growing cells in increasing concentrations of selection drugs it is possible to amplify significantly the copy number of the cotransfected gene of interest and concomitantly elevate the amount of protein produced (24).

The generation of stable cell lines, particularly the selection of amplified and high-expressing clonal cells, necessitates the screening of large numbers of transfected cells, both during the initial transfection and at each subsequent amplification step. This is mainly due to the wide variation in the level of expression and amplification of the transfected gene, depending on the chromosomal site of plasmid integration (25–27). For example, in CHO cells, gene amplification frequency in one transformant was 100-fold that of the others, and in another, amplification of transfected genes inserted near a centromere resulted in chromosome instability and rearrangements (25). These observations have theoretical implications for the method of gene introduction into mammalian cells. Often the gene of interest and the selectable gene marker (or, in the case of antibodies, the heavy and light chains) are located on two separate plasmids. These are cotransfected into the host cell where they recombine and integrate as a unit into the host chromosome (28). It is possible, therefore, that the two genes integrate in separate chromosomal loci of different transcriptional activity, necessitating the screening of large numbers of transfected cells. This potential problem may be resolved by placing both genes of interest on a single vector. To date, however, there is no firm evidence that a single expression vector is advantageous over two vectors. In the case of antibody expression, it has been concluded that equivalent levels of production and stability of the resulting cell lines have been obtained using single- and double-vector systems (19).

An alternative strategy for the efficient preparation of stable cell lines is site-specific gene integration using recombination systems such as Cre/loxP (29) and FLP/FRT from yeast (30). Cre (cyclization recombination) recombinase of bacteriophage P1 recombines DNA at 34-bp sites called loxP (locus of crossover of P1). The FLP recombinase from the 2-μm circle of Saccharomyces cerevisiae recognizes FRT (the FLP recombination target). It should be possible to engineer a cell line using a reporter gene to select a transcriptionally active chromosomal locus. Such a cell line could then be used for the routine excision and replacement of the reporter construct with the gene of interest. Progress in this area is ongoing (31,32), and a commercially available vector/host system makes use of the FLP/FRT elements (pOG vector, Stratagene, La Jolla, CA).

Recently, a well-differentiated epithelial cell line, Madin–Darby canine kidney (MDCK), was shown to be capable of producing large amounts of protein (33). The cells were transfected with a plasmid containing the cytomegalovirus promoter controlling the expression of matrix metalloproteinase 13 (MMP13). The yield of MMP13 was 10 mg/liter of conditioned medium, an amount that rivals yields obtained from CHO amplification systems. The authors point out that the unusually high yield could be attributed partially to the properties of MDCK cells, since CHO cells transfected with the same vector yielded much less protein (33).

TABLE 3
Vectors for Gene Expression in Mammalian Cells

Vector	Promoter	Induction	Selection marker — Mammal	Selection marker — Bacteria	f1/M13 origin	Reporter/ Purification tag/epitope	MCS	Vector size (kbp)	Comments	Commercial source or reference[*]
pSVK3	SV40 Early			Amp	f1		8	3.9	Transient	Amersham Pharmacia (www.apblotech.com)
pSVL	SV40 Late			Amp			5	4.9	Transient	Amersham Pharmacia
pMSG	MMTV-LTR (mouse mammary tumor virus)	Dexamethasone	gpt	Amp			4	7.6	Transient/stable	Amersham Pharmacia
pCH110	SV40 Early			Amp			1	7.1	Promoter screen	Amersham Pharmacia
pTargeT	hCMV-IE[b] (cytomegalovirus Immediate early)		Neo	Amp	f1		10	5.7	Transient/stable	Promega (www.promega.com)
pSI	SV40 Early			Amp	f1		10	3.6	Transient	Promega
pCI	hCMV-IE			Amp	f1		11	4.0	Transient	Promega
pCI-neo	hCMV-IE		Neo	Amp	f1		9	5.5	Transient/stable	Promega
pOPRSVI/MCS	RSV-LTR (Rous Sarcoma virus)	IPTG[c]	Neo	Amp	f1		8	5.6	Requires cotransfection with pCMVLacI	Stratagene (www.stratagene.com)
pBK-CMV	hCMV		Neo	Kana	f1		17		Phagemid	Stratagene
pBK-RSV	RSV-LTR		Neo	Kana	f1		17		Phagemid	Stratagene
pCMV-Script	hCMV		Neo	Kana	f1		17	4.3		Stratagene
pDual	hCMV (mutated)		Neo	Kana	f1	CBP[d]		5.5	Mammalian and bacterial expression	Stratagene
pCMV-Tag series	hCMV		Neo	Kana	f1	FLAG[e], c-myc	1–13	~4.3	Transient	Stratagene
pFLAG series	hCMV			Amp	f1	FLAG				Sigma (www.sigma-aldrich.com)
pSFV1								11.0	Vector serves as template for In vitro synthesis of RNA	Life Technologies (www.lifetech.com)
pTet-Splice	Tet	Tetracycline		Amp	f1		4	5.2	Requires cotransfection with pTet-tTAk	Life Technologies
pTRE	hCMV*-1[f]	Tetracycline or doxycycline[g]		Amp			4	3.1	Requires cotransfection with pTet-On or pTet-Off	Clontech (www.clontech.com)
pRev-TRE	hCMV*-1	Tetracycline or doxycycline[g]	Hyg	Amp			6	6.5	Requires cotransfection with pRevTet-On or pRevTet-Off	Clontech
pRetro-On pRetro-Off	hCMV*-1	Tetracycline or doxycycline[g]	Puro	Amp			3	6.8 7.0	Do not require cotransfection with other vectors	Clontech
pLNCX	hCMV-IE		Neo	Amp			3	6.6	Retroviral vector (Transient/stable)	Clontech
pLXSN	5' LTR		Neo	Amp			4	5.9	Retroviral vector (Transient/stable)	Clontech
pLXIN	5' LTR		Neo	Amp			3	6.1	Retroviral vector (Bicistronic)	Clontech
pSIR	5' LTR		Neo	Amp			4	8.1	Retroviral vector (Self-inactivating)	Clontech
pLAPSN	5' LTR		Neo	Amp		AP[h] (fusion)		7.9	Retroviral vector (Transient/stable)	Clontech
pIRES-bleo/hyg/ neo/puro	hCMV-IE		Bleo, hyg, neo, puro	Amp			5–9	5.3–5.7	Bicistronic vector	Clontech
pIRES-EGFP pIRES-EYFP	hCMV-IE			Amp		EGFP[i] EYFP[j]	5	5.2	Bicistronic vector	Clontech

Vector	Promoter	Induction	Selection (mammalian)	Selection (bacterial)	Phage origin	Tag/epitope	MCS frames	Size (kb)	Features	Source
pCDM8	hCMV			supF	M13		6	4.4	Transient	Invitrogen (www.invitrogen.com)
pcDNA1.1	hCMV			supF	M13		12	4.0	Transient	Invitrogen
pcDNA1.1/Amp				Amp			11	4.8		
pcDNA3.1-neo/zeo/hyg	hCMV		Neo, Zeocin, Hyg	Amp	f1		15	5.0–5.6	Stable	Invitrogen
pcDNA3.1/His/Myc-His/V5-His	hCMV		Neo	Amp	f1	(His)$_6$, c-myc, V5	11–13	5.5	Stable	Invitrogen
pRc/CMV2	hCMV		Neo	Amp	f1		6	5.5	Stable	Invitrogen
pRc/RSV	RSV-LTR		Neo	Amp	f1		6	5.2	Stable	Invitrogen
pSecTag2	hCMV		Zeocin, Hyg	Amp	f1	(His)$_6$, c-myc	12, 15		Igκ leader for protein secretion	Invitrogen
pSecTag2/hyg										Invitrogen
pDisplay	hCMV		Neo	Amp Kana	f1	c-myc, HA	8	5.3	Igκ leader, PDGFR TM domain for surface display	Invitrogen
pZeoSV2	SV40		Zeocin (P$_{CMV}$)	Zeocin (P$_{EM-7}$)	f1		17	3.5	Transient/stable	Invitrogen
pREP series	RSV-LTR		Hyg, HisD, Neo	Amp			7–9	9.5–11.8	EBV oriP and nuclear antigen (EBNA-1) for episomal replication	Invitrogen
pCEP4	hCMV-IE		Hyg	Amp			9	10.4	EBV oriP and nuclear antigen (EBNA-1) for episomal replication	Invitrogen
pEBVhis	RSV-LTR		Hyg	Amp		(His)$_6$, Ab	8	10.3	EBV oriP and nuclear antigen (EBNA-1) for episomal replication	Invitrogen
pcDNA4/HisMax	hCMV		Zeocin (P$_{SV40}$)	Zeocin (P$_{EM-7}$)	f1	(His)$_6$, Ab	12	5.3	Contains translational enhancer QBI SP163	Invitrogen
pVP22/myc-His	hCMV		Neo	Amp	f1	c-myc, (His)$_6$	8	6.4	VP22 fusions for nuclear targeting	Invitrogen
pIND series	ΔHSP (Heat shock protein)	Ecdysone	Neo, Hyg	Amp	f1	V5, (His)$_6$	16	5.0–5.7	Requires cotransfection with pVgRXR	Invitrogen
pSin series	SG (Sindbis subgenomic promoter)			Amp		(His)$_6$, Ab	6		Viral vector	Invitrogen
pEF series	hEF-1α (Elongation factor 1α)		Neo	Amp	f1	c-myc	4, 5		Targeting to cytoplasm, nucleus, mitochondria, ER	Invitrogen
pCMV series	hCMV		Neo	Amp	f1	c-myc	4, 5		Targeting to cytoplasm, nucleus, mitochondria, ER	Invitrogen
pTracer series	hCMV-IE, SV40, hEF-1α		Zeocin (P$_{CMV}$)	Zeocin (P$_{EM-7}$)	f1	GFP	9–13	4.2–6.2	Monitor transfection status by fluorescent microscopy	Invitrogen
pGT series									Modular bicistronic plasmids for gene therapy	InvivoGen (www.invivogen.com)
pCMV-LIC	hCMV-IE			Amp	f1			4.6		
pBacMam-1	Hybrid: hCMV-IE/avian actin			Amp	f1		6	11.6	Transient, stable by cotransfection	PharMingen (www.pharmingen.com)
pBacMam-2				Amp			8	7.6	Baculovirus-based vector for mammalian cells	Novagen (www.novagen.co.n)
pPOP	mPGK/lacO (phosphoglycerate kinase)	IPTG	Neo				8			

(179)

TABLE 3—*Continued*

Vector	Promoter	Induction	Selection marker Mammal	Bacteria	f1/M13 origin	Reporter/ Purification tag/epitope	MCS	Vector size (kbp)	Comments	Commercial source or reference[a]
pEF-LAC	hEF-1α/lacO	IPTG	Neo							(180)
pEF-BOS	hEF-1α									(181)
pBPVMT1	mMT-I (metallothionein I)	Cd²⁺, Zn²⁺, PMA[k]								(49)
pMT	hMT-II (metallothionein II)	Cd²⁺, Zn²⁺, PMA	Neo							(182)
pMT302	hMT-IIA (mutant)	Cd²⁺, Zn²⁺, PMA							High inducibility, low basal activity	(51)
pIFP	hIFN-α (Interferon-α)	Viruses	Neo, dhfr	Amp			2	8.9		(183)
pGRE5	5XGRE/Ad2MLP (Glucocorticoid response element/ adenovirus major late promoter)	Dexamethasone	Neo	Amp			7	4.0		(184)
pRDB	DRE/MMTV (dioxin response element)	TCDD[l]	Neo	Amp					5- to 10-fold inducibility	(185)
pLTRpoly	MoMLV-LTR (Moloney murine leukemia virus)		Neo	Amp			10	4.3		(186)
pKX	hLS (Leukosialin (CD43))			Amp					Selective ? T cells	(187)
pMU1	mU1a, U1b snRNA (small nuclear RNA)									(188)
pSRα	Hybrid: SV40/HTLVI-LTR									(189)
pRD133	CMV/Ad2TPL (adenovirus tripartite leader)			Amp			18			(190)
pHEKneo	κ Light chain		Neo	Amp					Selective for myeloma cells	(191)
pTIF-1	Variable heavy chain		Neo						Ig expression in lymphoid cells	(192)
pIE	HSV (Herpes simplex virus)								Requires cell line expressing HSV VP16 transactivator	(193)
p753	hUbiquitin C									(43, 44)
pEPI-1	CMV		Neo	Kana				6.7	Non-viral-based vector for stable episomal replication	(23)

[a] References for commercially available vectors may be found in the respective company catalogs.

[b] h, human; m. murine.

[c] Isopropyl-β-D-thiogalactopyranoside.

[d] Calmodulin-binding peptide.

[e] Synthetic peptide sequence DYKDDDDK.

[f] Compound promoter consisting of the tetracycline-responsive element (TRE), which contains seven direct repeats of the 42-bp *tetO* sequence and the minimal immediate early promoter of CMV, which lacks the CMV enhancer. This promoter is silent unless activated by (r)tTA.

[g] Induction or repression, depending on the cotransfected vector.

[h] Alkaline phosphatase.

[i] Enhanced green (yellow) fluorescent protein.

[j] Synthetic promoter.

[k] 4-β-Phorbol 12-myristate 13-acetate.

[l] 2,3,7,8-Tetrachlorodibenzo-p-dioxin.
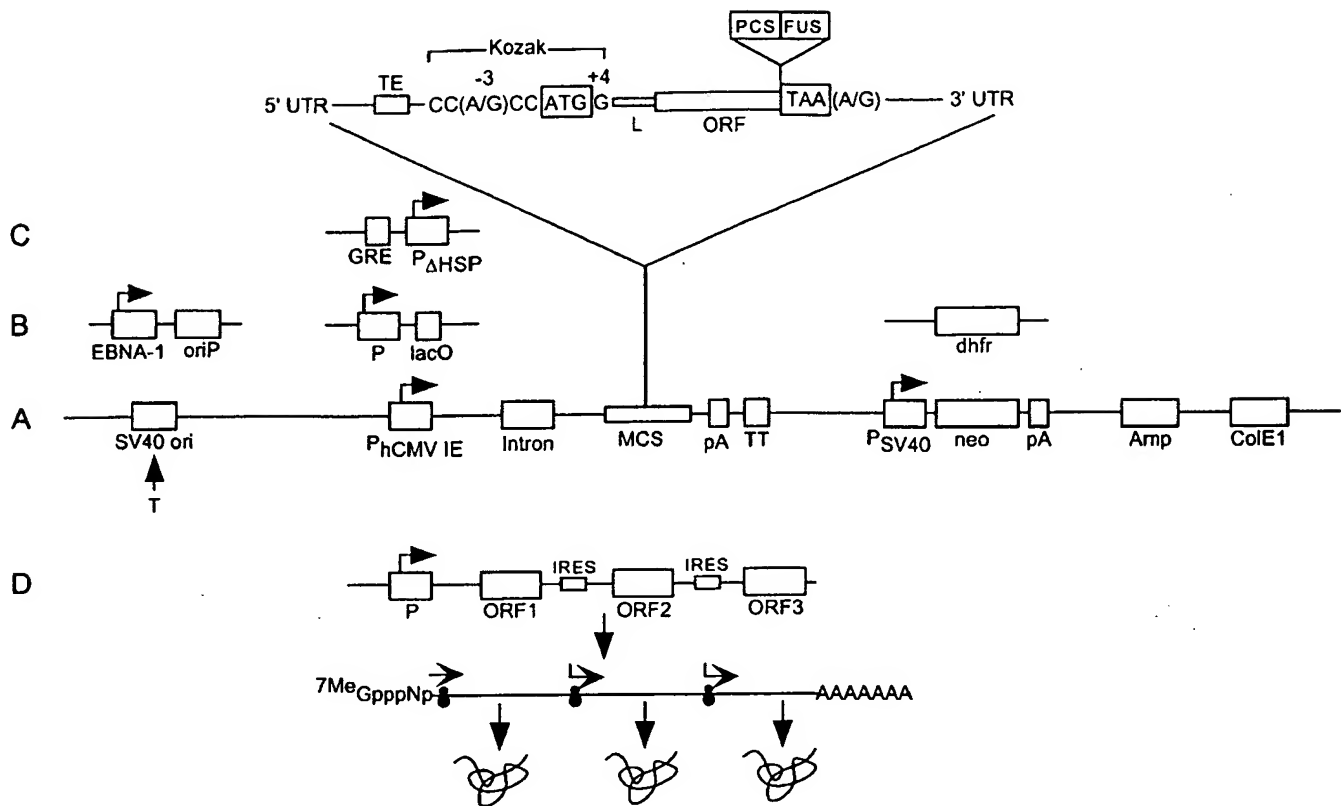
FIG. 1. Configuration of genetic elements in monocistronic (A) and polycistronic (D) expression vectors. Specific elements are shown for illustrative purposes and are not drawn to scale. The source, position, and combination of different components in the vector may vary in order to meet specific experimental requirements. SV40 ori is required for transient gene expression in COS cells. EBNA-1 and oriP facilitate high-copy episomal replication in primate and canine cell lines. The various promoter (P) elements allow constitutive (A) or inducible (B, C) expression. The optimal translational initiation sequence (Kozak) and termination codon followed by purines are shown. The ColE1 origin of replication and the ampicillin-resistance gene allow vector propagation in bacteria. The neomycin-resistance gene facilitates selection in mammalian cells, and the *dhfr* gene allows both selection and gene amplification. In a polycistronic vector (D) IRES elements allow multiple ORFs to be efficiently translated from a single transcript. See text for details. Amp, ampicillin resistance gene; ColE1, prokaryotic origin of replication; dhfr, dihydrofolate reductase; EBNA, Epstein-Barr virus nuclear antigen; FUS, fusion moiety; GRE, glucocorticoid response element; hCMV IE, human cytomegalovirus immediate early enhancer/promoter; HSP, heat shock protein; IRES, internal ribosome entry site; lacO, *lac* operator; L, leader (targeting sequence); MCS, multiple cloning site; neo, neomycin resistance gene; ORF, open reading frame; ori, origin of replication; oriP, Epstein-Barr virus origin of replication; P, promoter; pA, polyadenylation signal; PCS, protease cleavage site; T, SV40 large tumor (T) antigen; TE, translational enhancer; TT, transcription terminator; UTR, untranslated region.

3. TRANSCRIPTIONAL CONTROL ELEMENTS

Promoters and Enhancers

Although the physical boundaries between these two control elements are not always clear, promoters are operationaly defined as the site of transcription initiation, an event mediated through interactions of transcription factors with their cognate promoter and enhancer elements (34–36). Enhancers potentiate promoter activity, temporally as well as spatially (34,37). In general, promoters contain the TATA box, located upstream of the transcription initiation site, and the CAAT box, located upstream of the TATA box. Both regions bind transcription factors that facilitate transcription initiation; however, there are promoters that do not contain a TATA box (38,39).

Many promoters are transcriptionally active in a wide range of cell types and tissues. However, most exhibit tissue specificity, a property that must be care-

fully considered prior to the construction and use of expression vectors (40,41). For example, the widely used cytomegalovirus promoter exhibits low transcriptional activity in hepatocytes (42). Strong constitutive promoters which drive expression in many cell types include the adenovirus major late promoter, the human cytomegalovirus immediate early promoter, the SV40 and Rous Sarcoma virus promoters, and the murine 3-phosphoglycerate kinase promoter. The human ubiquitin C promoter is active in tissue culture (43), and it is capable of high-level gene expression in a very broad range of tissues (44).

Tissue specificity of promoters is of particular interest in gene therapy applications. An interesting strategy was recently devised to enhance the transcriptional activity of weak promoters without loss of tissue specificity (45). The principle behind this strategy was to use a cell type-specific promoter to drive the simul-

TABLE 4

Selection Markers for Gene Expression in Mammalian Cells

Gene	Phenotype	Action of selective agent	Reference
	Positive selection		
dhfr (dihydrofolate reductase)	Resistance to MTX[a]	MTX is a competitive inhibitor of DHFR	194, 195, 196
xgprt (gpt) (xanthine–guanine phosphoribosyl transferase)	Xanthine as the source for guanine synthesis	Aminopterin and mycophenolic acid in dialyzed medium block *de novo* synthesis of GMP	197
aph (neo) (aminoglycoside phosphotransferase)	Resistance to G418[b]	G418 blocks mammalian protein synthesis	198, 199
hph (hyg) (hygromycin-B-phosphotransferase)	Resistance to hygromycin B	Hygromycin B blocks protein synthesis	200, 201
pac (puro) (puromycin-N-acetyl transferase)	Resistance to puromycin	Puromycin blocks protein synthesis	202, 203
ble (bleomycin)	Resistance to bleomycin, phleomycin, or zeocin	Bleomycin belongs to a group of related glycopeptide antibiotics which are believed to cause DNA strand scission	204, 205
hisD (histidinol dehydrogenase)	Resistance to histidinol	Histidinol is cytotoxic; HD oxidizes histidinol to histidine	206
trpB (tryptophan synthase (β subunit))	Indole as the source for tryptophan synthesis		206
atpA (Na$^+$, K$^+$-ATPase α subunit)	Resistance to ouabain	Ouabain belongs to a group of related cardiac glycosides which block transport of Na$^+$ and K$^+$ by intact cell membranes	207, 208
ada (adenosine deaminase)	Resistance to Xyl-A[c]	Xyl-A is converted to Xyl-ATP, which damages nucleic acids	209, 210
codA (cytosine deaminase)	Resistance to PALA[d]	PALA blocks *de novo* synthesis of pyrimidines; CD converts cytosine in the medium to uracil	211, 212
	Negative selection		
codA (cytosine deaminase)	Cell death	CD converts 5-fluorocytosine to 5-fluorouracil	213
HSV-TK (Herpes simplex virus thymidine kinase)	Cell death	TK phosphorylates the selection drug ganciclovir which incorporates into DNA; ganciclovir is a poor substrate for mammalian TK	214
	Positive or negative selection		
Fusion: hyg–tk	Positive: resistance to hygromycin Negative: cell death		215
Fusion: tk–neo	Positive: resistance to G418 Negative: cell death		216
Fusion: tk–bsd	Positive: resistance to blasticidin S Negative: cell death		217
Fusion: pac–tk	Positive: resistance to puromycin Negative: cell death		218
Fusion: hyg–codA	Positive: resistance to hygromycin Negative: cell death		218
Fusion: codA–neo	Positive: resistance to G418 Negative: cell death		218
Fusion: codA–bsd	Positive: resistance to blasticidin S Negative: cell death		218
Fusion: pac–codA	Positive: resistance to puromycin Negative: cell death		218

[a] Methotrexate. Stable cell lines may be established using DHFR-deficient Chinese hamster ovary cells and a normal *dhfr* gene or wild-type cells and a mutant *dhfr* encoding an enzyme resistant to MTX.

[b] G418 is an aminoglycoside, similar in structure to neomycin.

[c] 9-β-D-Xylofuranosyl adenine.

[d] *N*-(phosphonacetyl)-L-aspartate.

taneous expression of the gene of interest and an artificial transcriptional activator to stimulate transcription through binding sites in the promoter. This "positive feedback loop" was achieved using a fusion transcription factor composed of the Herpes simplex virus VP16 transcriptional activation domain and the DNA-binding domain of LexA. It was shown that the transcriptional activity of two different promoters was increased 14- to 100-fold while maintaining cell type specificity (45).

Promoters can be divided into two classes, those that function constitutively and those that are regulated by induction or derepression. Inducible promoters are desirable for the production of proteins that may be toxic to the host cell, such as diptheria toxin (46), for the study of gene regulation during development in transgenic animals (13,47), and for experimental and therapeutic applications of gene transfer (48). Promoters used for high-level production of proteins in mammalian cells should be strong and, preferably, active in a wide range of cell types to permit qualitative and quantitative evaluation of the recombinant protein. Inducible promoters should exhibit a minimal level of basal transcriptional activity and be capable of substantial induction with a nontoxic inducer in a simple and cost-effective manner.

The widely used metallothionein promoter exhibits high basal expression level and a poor induction ratio (49). Moreover, heavy metals used to induce this promoter are cytotoxic. Two groups have addressed the high basal expression of the human MTIIA promoter. In one study, the substitution of multiple metal response elements for a region involved in basal expression caused up to 200-fold inducibility of the promoter (50). In the other study, mutation of specific nucleotides within the promoter resulted in low basal activity and a 13- to 35-fold induction ratio, depending on the cell line (51).

The functionality of the bacterial *lac* operator–repressor system in mammalian cells (52,53) has been exploited for the inducible expression of heterologous genes in mammalian cells. A potential advantage of this system over the use of endogenous cellular transactivators is that the *lac* operator, the recognition sequence for the *lac* repressor, occurs at low frequency in mammalian cells (54) and should facilitate high specificity in target gene regulation. In addition, the *lac* repressor has an extremely high affinity for the *lac* operator—the dissociation constant is about 10^{-13} M (55). Different versions of this system exhibited 1200-fold (56) or 10,000- to 20,000-fold (57) induction of gene expression, with no detectable expression in the absence of inducer and a high specificity for the gene under study. However, the inducer isopropyl β-D-thiogalactopyranoside (IPTG) is cytotoxic, albeit at high concentration (50 mM) (53). IPTG concentrations typically used for induction of mammalian expression systems range from 0.1 to 5 mM. The toxicity of IPTG has precluded its use in the large-scale production of therapeutic proteins. Several studies have described the use of temperature-sensitive *lac* repressors for use in mammalian cells (57) or in *Escherichia coli* (58–60). The bacterial thermoinducible *lac* repressor systems should be adaptable for use in mammalian cells. Recent advances in the construction of mammalian inducible expression systems have been reviewed (8–13).

Introns

Most genes from higher eukaryotes contain introns which are removed during RNA processing. Genomic constructs have been shown to be expressed more efficiently in transgenic animals than identical constructs lacking introns (61,62). Although many cDNA constructs lacking introns can be expressed efficiently in mammalian cells, Buchman and Berg (63) showed that the inclusion of introns leads to a 10- to 20-fold increase in expression, and some sequences, such as the β-globin cDNA, show a virtual requirement for the presence of an intron. The placement of introns at the 3' end of the transcription unit has been reported to lead to aberrant splicing (64,65); therefore, it is preferable to place introns at the 5' end of the open reading frame. Another caveat involves the use of the SV40 19S late mRNA intron, which appears to be inappropriate for the production of antibodies (66). A synthetic intron SIS generated by the fusion of an adenovirus splice donor site and an immunoglobulin G splice acceptor site was very active in a variety of cell types (67). In addition to their ability to increase gene expression, introns have been used in plasmid constructions in order to facilitate gene expression in both mammalian and yeast (68) or mammalian and bacterial cells (69).

Polyadenylation Signals

Most eukaryotic nascent mRNAs possess a poly(A) tail ($n \approx 200$) at their 3' ends, which is added during a complex process that involves cleavage of the primary transcript and a coupled polyadenylation reaction (70). The poly(A) tract is important for mRNA stability and translatability (71,72). The signals for polyadenylation of mammalian mRNAs are well defined: One component consists of a highly conserved AAUAAA sequence which is located about 20–30 nucleotides upstream of the 3' end of the mRNA, and the other element consists of an unconserved GU-rich sequence immediately downstream of the polyadenylation site (73,74). There are several efficient poly(A) signals to use in mammalian expression vectors, including those derived from bovine growth hormone (75), mouse β-globin (76), the SV40 early transcription unit (77), and the Herpes simplex virus thymidine kinase gene (78).

Transcription Terminators

Continued transcription from an upstream promoter through a second transcription unit inhibits the function of the downstream promoter, a phenomenon known as promoter occlusion or transcriptional interference. This event has been described in both prokaryotes (79) and eukaryotes (80,81). The proper placement of transcriptional termination signals between two transcription units can prevent promoter occlusion (81). Prokaryotic transcription terminators are well characterized, and their incorporation in expression vectors has been shown to have multiple beneficial effects on gene expression (reviewed in 82). In eukaryotes, a consensus sequence consisting of ATCAAA(A/T)TAGGAAGA has been identified in the termination region of nine genes (83).

4. TRANSLATIONAL CONTROL ELEMENTS

The optimal expression of eukaryotic cDNAs requires the careful consideration of several structural features, including the 5′ and 3′ untranslated sequences and the nucleotide context around the translation initiation codon (the Kozak sequence). In addition, codon usage may have a substantial impact on the translation efficiency of some genes in mammalian cells.

Kozak Sequence

Using systematic mutagenesis of specific genes as well as comparison of eukaryotic mRNA sequences, Kozak (84) defined the optimal translation initiation sequence in eukaryotic mRNAs. CC(A/G)CCaugG emerged as the consensus sequence for initiation in higher eukaryotes. The purines A or G in position −3 (i.e., three nucleotides upstream from the AUG codon) and G immediately following the AUG codon are the most influential in facilitating optimal translation initiation.

5′ Untranslated Region

In eukaryotic cells translation of most mRNAs is initiated according to the "scanning model" (85). The initiation complex, consisting of the 40S ribosomal subunit and cap-binding proteins, forms at the mRNA 5′ terminal cap (m⁷GpppN) followed by movement of the ribosome to the "correct" initiating AUG codon in a favorable sequence context (84). The presence of AUG codons in the 5′ untranslated region (5′ UTR) of the transcript can severely depress translational initiation at the "authentic" start codon, although the extent of inhibition depends on sequences surrounding the upstream AUG (86,87). Such inhibition can be minimized by the presence of a translation termination codon in-frame with the upstream AUG (86,87). An addi-

tional concern involves the potential of the 5′ UTR to form extensive secondary structure. Thus, GC-rich regions have the potential to form stable hairpin structures which can inhibit translation initiation, a phenomenon that has been extensively documented in eukaryotic (88–90) and prokaryotic expression systems (reviewed in 82). One solution to these potential problems is the removal of the 5′ UTR prior to the insertion of cDNAs into expression vectors, with the caveat that the 5′ UTR may contain translational enhancer elements, such as the SP163 element of the vascular endothelial growth factor mRNA (91). The SP163 sequence has been shown to enhance the translation of different mRNAs 25- to 40-fold in several mammalian cell types (91).

3′ Untranslated Region

mRNA destabilization can be effected by specific sequences present in the 3′ UTR. This topic is discussed in section 5 (mRNA Stability). In addition, translational regulation of certain mRNAs is mediated by protein-binding AU-rich elements located in the 3′ UTR (92).

Termination Codon

Experimental evidence indicates that translational termination in mammalian genes may be modulated by nucleotides additional to those of the trinucleotide stop codon. Statistical analysis of the context of termination codons in 5208 mammalian genes showed a highly significant bias in the position immediately following the stop codon (Fig. 1 in 93). The significance of this bias in translational termination was tested in both *in vivo* and *in vitro* assays, and it was determined that the base following the stop codon influences the efficiency of translation termination. Thus, tetranucleotides with a purine in the fourth position are more effective as termination signals than those with a pyrimidine (93).

Codon Usage

Both prokaryotic and eukaryotic genes exhibit a nonrandom usage of synonymous codons (94,95). In general, highly expressed genes exhibit a greater degree of codon bias than do poorly expressed ones, and the frequency of use of synonymous codons usually reflects the abundance of their cognate tRNAs (96). Most studies of codon optimization for gene expression have been carried out in *E. coli* (reviewed in 82,97). *E. coli* exhibits a highly biased codon usage and, therefore, the possibility exists that heterologous genes enriched with codons that are rarely used by *E. coli* (98) may not be expressed efficiently in *E. coli*. Similarly, it is possible that mammalian codon usage may affect transla-

tion efficiency of heterologous genes, as documented below.

As part of gene transfer studies for the correction of human genetic disorders, Wheeler *et al.* (99) studied the mitochondrial enzyme ornithine transcarbamylase (OTC). The OTC gene was synthesized by PCR using codons optimized for mammalian mitochondrial as well as for universal codon usage. The synthetic OTC gene was successfully expressed in *E. coli*. However, transient transfections of COS-7 cells failed to produce enzymatic activity or immunoreactive OTC protein, despite the detection of mRNA specific for the synthetic OTC gene and the successful transient expression of an unmodified human OTC gene (99). It is likely that OTC mRNA could not be translated by the mammalian cytoplasmic tRNA pool, a supposition that potentially could be proved by the use of a mitochondrial *in vitro* translation system. The jellyfish *Aequorea victoria* green fluorescent protein (GFP) is widely used as a reporter in many gene transfer applications, including gene therapy. Different versions of the GFP gene optimized for human codon usage have been shown to exhibit significantly higher expression levels (4- to 10-fold) and increased fluorescence intensity in mammalian cells (100,101). It is possible, however, that the altered codon content may have stabilized the GFP mRNA, in addition to enhancing its translational efficiency. In another example of codon optimization, a GFP gene modified to contain synonymous codons from highly used human genes showed a 20-fold higher expression level in maize leaf cells than in the original GFP sequence (102).

Codon optimization may also have a significant impact in vaccination studies. A sequence from the human immunodeficiency virus type 1 gp120 gene was optimized using codons from highly expressed human genes, resulting in higher expression levels (103,104). It was shown that the increase in efficiency of expression was not due to enhanced mRNA stability (103). The difference in expression levels between the codon-optimized and wild-type constructs depended on the vector/host combination used. Thus, in 293T cells transiently transfected with the vector pCdm7, there was a 10- to 50-fold increase in expression levels with the synthetic gene. Moreover, immunization of BALB/c mice with the same codon-optimized DNA resulted in significantly increased antibody titer and cytotoxic T-lymphocyte reactivity, suggesting a correlation between expression levels and the immune response (104). Similar observations have been obtained with a different pathogen, *Listeria monocytogenes.* Codon-optimized plasmid DNA sequences showed substantially higher expression levels in mammalian cells and conferred partial protection against listerial infection in mice (105).

5. mRNA STABILITY

The turnover of mRNA is an important posttranscriptional mechanism for the physiological control of gene expression (106). The short half-life of some mRNAs, such as cytokines, cell cycle control factors, and oncogenes, is thought to permit the rapid cessation of protein production in response to rapidly changing physiological conditions. Conversely, the high degree of stability of some mRNAs, such as the globins, collagens, and crystallins, ensures their accumulation to high steady-state levels following an increase in transcription (107). Thus, the recognition that the metabolic stability of mRNA can have profound effects on gene expression has led to specific suggestions for potential therapeutic interventions (107). The potential ability to extend significantly the half-life of transcripts offers an attractive means of enhancing protein production in mammalian expression systems.

One determinant of eukaryotic mRNA lability is an AU-rich sequence in the 3' UTR of many unstable mammalian mRNAs (108–110). The insertion of an AU-rich element into the 3' UTR of a stable mRNA destabilizes the chimeric transcript (109,111). The optimal sequence for this destabilizing determinant is believed to be UUAUUUAUU (111) or UUAUUUA(U/A)(U/A) (112). The removal of these sequences from the 3' UTR of unstable mRNAs is desirable for maximal protein production.

Synthetic 5' secondary structures have been shown to increase mRNA half-lives in *E. coli* (113). In seeking to maximize transcript stability and protein production in mammalian cells, investigators have substituted the UTRs of stable mRNAs, such as β-globin, for the UTRs of transcripts of interest (e.g., 114,115). This strategy, effective in specific cases, may not have universal application, as mRNA degradation is effected by multiple pathways in mammalian cells (116,117). Thus, in addition to exonucleolytic activity at both the 5' and 3' termini, determinants of mRNA half-life have been mapped to the coding regions of several mRNA species (106,117,118). In this case, the addition of a stabilizing UTR probably will have no effect on transcript stability. Furthermore, mRNA stability is modulated by a variety of cell-specific proteins that act *in trans* to destabilize (119,120) or stabilize transcripts (121–123). The use of a specific UTR for the purpose of stabilizing a heterologous transcript in mammalian cells assumes the presence of the cognate UTR-binding proteins in the same cells. At present, our knowledge of the distribution of such proteins in different mammalian cell lines used for protein production is incomplete (107,120).

It would be a significant omission not to mention that levels of heterologous proteins are also affected by protein degradation pathways. This is an important topic, beyond the scope of this review. Strategies for

TABLE 5

Fusion Moieties for Gene Expression in Mammalian Cells[a]

Fusion partner (source)	Ligand/substrate	Detection	Application	Reference[b]	Commercial source of expression vector[c]
FLAG peptide	Anti-FLAG monoclonal antibodies M1 and M2	Antibody	Purification, detection	219, 220	Stratagene (www.stratagene.com)
(Histidine)₆	Ni²⁺-nitrilotriacetic acid	Antibody	Purification, detection	221, 222	Invitrogen (www.invitrogen.com) Qiagen (www.qiagen.com)
Glutathione S-transferase (Schistosoma japonicum)	Glutathione	Biochemical, antibody	Purification, detection	223, 224, 225	Amersham Pharmacia (www.apbiotech.com)
c-myc epitope	Antibody	Antibody	Purification, detection		Invitrogen
Calmodulin-binding peptide	Calmodulin		Purification		Stratagene
Fc-Hinge	Protein A		Purification, protein dimerization, higher protein yield, longer protein half-life	226, 227	
IgG1 and IgM heavy chain constant regions			Longer protein half-life	228	
Streptococcal protein G	Serum albumin		Purification, longer protein half-life	229	
Serum albumin			Longer protein half-life	229, 230[d]	
Viral glycoprotein transmembrane domain			Surface expression for vaccination	231	
Platelet-derived growth factor receptor (PDGFR) transmembrane domain			Surface expression for ligand-binding interactions		Invitrogen
Herpes simplex virus glycoprotein D (gD) domain	Antibody	Antibody	Purification	232	
Epstein-Barr virus nuclear antigen 1 GGAGAGAG			Longer protein half-life	125	
Growth hormone (human, rat)	Antibody	Immunoassay	Detection, monitor promoter activity	233, 234	
Alkaline phosphatase (mammalian/bacterial)	p-Nitrophenyl phosphate	Electrochemical, chemiluminescence, fluorescence	Detection, monitor promoter activity, biosensors	143, 234, 235, 236	Clontech (www.clontech.com)
β-Galactosidase (Escherichia coli)	β-Galactosides	Electrochemical, chemiluminescence, fluorescence	Detection, monitor promoter activity, biosensors	143, 234, 237	Clontech
Chloramphenicol acetyltransferase (E. coli)	Chloramphenicol or its derivatives	Radioisotope, fluorescence	Detection, monitor promoter activity, biosensors	143, 234, 238, 239	Promega (www.promega.com)
Luciferase (Photinus pyralis) (Luciola mingrelica)	Firefly luciferin	Bioluminescence	Detection, monitor promoter activity, biosensors	143, 234, 240–242	Promega
Luciferase (Vibrio harveyi)	n-Decyl aldehyde	Bioluminescence	Detection, monitor promoter activity, biosensors	143, 240[e]	
Luciferase (Renilla reniformis)	Coelenterazine	Bioluminescence	Detection, monitor promoter activity, biosensors	243	
Green fluorescent protein and its variants (Aequorea victoria)		Fluorescence	Detection, monitor promoter activity, higher protein yield, biosensors	143, 144, 244, 245, 246	Clontech, Invitrogen
Aequorin (A. victoria)	Coelenterazine	Bioluminescence	Immunoassay, hybridization assay, Ca²⁺ reporter	143	

[a] This table does not include heterologous signal sequences or antibody variable regions used for protein targeting.
[b] References indicate application of fusion moieties in mammalian cells and do not necessarily reflect the original development of said fusion partner.
[c] No effort has been made to provide a complete list of commercial suppliers.
[d] Expression of albumin–CD4 fusion in yeast.
[e] Low expression of bacterial luciferase in mammalian cells, with an increase of more than 10-fold when cells were grown at 30°C.

minimizing protein degradation in prokaryotes have been reviewed (82,124). In contrast, less light appears to shine on this complex issue with regard to mammalian protein production. It is worthwhile to point out an interesting recent study on ubiquitinated proteins (125,126) that has implications for the modulation of protein degradation in eukaryotes. The insertion of a minimal eight-residue glycine–alanine repeat into a protein that is targeted for proteolysis via the ubiquitin–proteasome pathway inhibited its degradation.

6. POLYCISTRONIC MESSAGES

The scanning model of translation initiation (see section 4) does not apply to many viral (127,128) and apparently, some cellular messages (91,129). These are translated in a cap-independent manner at internal

sites known as internal ribosome entry sites (IRES). It is believed that cellular *trans*-acting proteins bind to the IRES element and facilitate ribosome binding and translational initiation (130). However, the precise mechanism of IRES-mediated translation is unclear. In a critical examination of published studies on IRES elements, Kozak (130a) concluded that recent concepts about the mechanism of internal translation initiation at putative IRES complexes are premature. Moreover, the experimental evidence for the presence of IRES elements in cellular mRNAs from mammals has been challenged (130a). Similarly, reports of internal initiation in yeast have been questioned (130b). The reader is referred to Kozak (130a) for a detailed discussion of this topic.

Earlier designs of polycistronic constructs for the expression of two or more genes from a single transcript had several limitations, discussed previously (131,132). Robust polycistronic vectors now utilize IRES elements that facilitate internal ribosome binding to the second and subsequent transcription units (128,133,134). Vectors containing IRES elements have a variety of applications: (1) establishment of stable mammalian cell lines which requires coexpression of the gene of interest and a selectable marker (135,136); (2) efficient gene amplification in the generation of stable cell lines (137); (3) clonal selection of cells expressing inducible gene products (138); (4) characterization of antibody responses in DNA immunization protocols (139); and (5) coexpression of genes for positive–negative (suicide) selections in gene therapy. For example, the multidrug resistance gene *MDR1* has been coexpressed with the Herpes simplex virus thymidine kinase (TK) gene (140). The TK gene acts both as a selectable marker in TK-deficient cells and as a suicide gene. Thus, cells expressing the TK gene can be selected against using the nucleoside analog ganciclovir. Additional applications of IRES elements include (6) gene trapping for the identification of developmentally regulated genes (134); (7) gene targeting (134,141); and (8) coordinated constitutive or adjustable high-level expression of three genes in mammalian cells (142).

7. FUSION MOIETIES

Their wide range of applications makes fusion components valuable tools in both prokaryotic and eukaryotic gene expression systems (82). Fusion moieties (Table 5) can be used as affinity handles for the facile isolation and purification of proteins, as reporter genes for the study of promoter activity or localization of proteins in cellular compartments, as protein dimerization domains, to increase expression, solubility, and secretion of proteins, or to display polypeptides on the surface of cells for vaccine development, protein–protein interactions, drug screening, and other potential applications. Fusion constructs have also been used to increase the half-life of target proteins for potential therapeutic applications (Table 5). In recent years, the fusion of reporter genes to heterologous promoters is being actively pursued for the engineering of bacterial biosensors in analytical, environmental, and clinical research (143,144). Many of the reporter genes used in bacterial biosensors should have applications in mammalian expression systems.

The design of protease cleavage sites between the fusion moiety and the target protein facilitates the separation of the two components. Technical issues pertaining to site-specific proteolysis of fusion proteins have been reviewed (145). The design of fusions for protein targeting to specific cellular compartments has been reviewed (146,147).

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EXHIBIT 11

Status of gene therapy for cystic fibrosis lung disease

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Gene therapy for the treatment of cystic fibrosis should be a "natural": Cystic fibrosis (CF) is a recessive disease associated with loss of function mutations in the CF transmembrane conductance regulator (CFTR) gene, which has a well-characterized gene product; heterozygotes, as predicted, appear to be phenotypically perfectly normal; the level of expression of CFTR in affected cells generally appears to be low; and the dysfunctional epithelial lining cells in the organ most affected by CF (the lung) are available for direct vector delivery via topical administration (1). However, despite an impressive amount of research in this area, there is little evidence to suggest that an effective gene-transfer approach for the treatment of CF lung disease is imminent. The inability to produce such a therapy reflects in part the learning curve with respect to vector technology and the failure to appreciate the capacity of the airway epithelial cells to defend themselves against the penetration by moieties, including gene-therapy vectors, from the outside world. This Perspective will focus on the issues that impact on moving this field forward.

What is the target for CF gene therapy in the lung? Cystic fibrosis affects the conducting airways of the lung and not the alveolar surfaces. The airways in general consist of a "large" airway (bronchial) region that is lined by a pseudostratified columnar superficial epithelium and contains numerous submucosal glands, and a "small" airway (bronchiolar) region that is lined by a simple columnar epithelium and is devoid of glands. Central issues for CF gene therapy are which region (large vs. small) and which tissue (superficial epithelium vs. glands) should be targeted.

Obviously, the answer to this question requires knowledge of the pathogenesis of CF lung disease. As reviewed earlier in this Perspective series (2, 3), this is a controversial issue. Although the so-called "isotonic" and "hypotonic" airway surface liquid theories have different predictions on the pathogenesis of CF airways infection, both agree that defects in the superficial epithelium may initiate CF lung disease. However, studies from other model cell culture systems, like Calu-3 cells and cultured gland acini (4), predict that there may be abnormalities in gland volume/compositional (HCO_3^-) regulation in CF that may be more important in the pathogenesis of CF airways infection. This debate can also be viewed in the context of the individual cell types in the airways. Advocates of the importance of the superficial epithelium in CF pathogenesis likely would favor targeting the ciliated cell, which clearly exhibits all of the ion transport functions of CFTR and exhibits abnormal

function in patients with CF (5), whereas advocates of the importance of the submucosal gland would likely favor targeting the submucosal gland serous cells, which may be the highest CFTR-expressing cell type in the lung (6).

In the absence of definitive data from model systems, from an operational point of view probably the best strategy is to examine the sequence of disease in young CF patients and select the target based on those data. Perhaps the most relevant observations are that CF infants typically present clinically with physical and roentgenographic findings of bronchiolitis, exhibit as their first pulmonary function abnormality small airways obstruction, and have evidence from autopsy studies of mucus plugs in small airways. These data suggest that as in other major airway diseases — chronic bronchitis, for example — small airways are the initial and major site of functional disease (airflow obstruction) in the CF lung. Therefore, restoration of function in the superficial epithelium lining small airways should be clinically beneficial. This reasoning does not dismiss expression of abnormal function in proximal CF airways. Indeed, virtually all studies of epithelial dysfunction in the lung have detected differences in this region, but the importance of small airways obstruction in the phenotype of airways disease suggests that selective correction of epithelial defects in the large airways will not be therapeutically useful. Interestingly, virtually all gene-therapy trials to date have delivered vectors via the topical route to the superficial epithelium, but it is not obvious that aerosol delivery strategies have been optimized for small airway deposition. Although deposition is difficult in patients with airways occluded by mucus plugs and infection, it will be important to develop efficient means to deliver vectors via aerosol to small airways.

However, it is possible that it may be important to treat submucosal glands and that we will not be able to devise strategies to effectively dose CF airways. Therefore, it would appear prudent to continue efforts to deliver vectors systemically that could access gland regions as well as the superficial epithelium of occluded airways.

How much gene transfer is enough? A key issue is to distinguish between the concepts of "level of CFTR transduced/cell" and "percent correction," denoting the fraction (percentage) of CF cells within an epithelial region (area) that are "corrected." With regard to level of transduced CFTR/cell, based on endogenous CFTR expression data it is likely that the level required for ciliated cells will be very low whereas the level required for

serous cells will be higher (4). With regard to percent correction, initial studies focused on this issue utilized monolayers of immortalized CF epithelial cells comprised of varying percentages of CF cells and CF cells "corrected" with wild-type CFTR (7). These studies showed that approximately 6%-10% of the cells within a monolayer must consist of "corrected" CF cells to restore normal Cl⁻ transport function.

While informative, the study by Johnson et al. emphasizes the importance of both knowledge of the pathogenesis of CF lung disease and the fidelity of the model system to the in vivo situation to accurately address this issue. For example, the epithelial model system used for these studies comprised a mosaic monolayer epithelium that was highly connected via gap junctions, utilized "corrected" CF cells that expressed rather high levels of CFTR per cell, and focused only on Cl⁻ transport. The "amplification of correction" (i.e., normalization of function with correction of a small percentage of epithelial cells) reported in that study likely reflected the movement of Cl⁻ ions from non-corrected to corrected cells through gap junctions, with Cl⁻ secretion reflecting exit through a "lot" of CFTR in a small number of corrected cells. It is likely that the number of gap junctions per cell in a well-differentiated epithelium in vivo is less than in the immortalized airway cells used in this study, and hence the percentage of cells requiring correction to restore normal Cl⁻ transport in vivo may well exceed 10%.

The relationship between normalization of function and percentage of corrected cells within the epithelium is also quite different if one considers Na⁺ transport. Recent data have suggested that lack of CFTR regulation of Na⁺ transport rates may be important in the pathogenesis of CF lung disease and that the relationship between CFTR and Na⁺ transport is more "local," i.e., may involve protein-protein interactions confined to single cells (1). Thus, when abnormal CF Na⁺ transport is used as an index of correction, one finds a linear relationship between the percentage of cells in a monolayer corrected and the percent normalization of function (8).

Consequently, the simplest strategy to assure efficacy is to mimic the normal pattern of expression, i.e., there should be a low level of expression per cell, and virtually every affected cell (100%) should be corrected. Is there an acceptable percentage below 100% of cells that might justify the initiation of a clinical trial? Given the likely difficulties in achieving gene transfer in man in vivo compared to any model system, certainly more than 10% of cells should be transduced in the most relevant model systems, e.g., studies of human explants and pertinent animal models in

vivo. Unfortunately, none of the current in vivo model systems, such as the CF mouse, yield a sufficiently accurate lung infection phenotype to allow this critical question to be evaluated in a whole animal system.

Where are we in the clinic? Approximately 20 trials of CF gene therapy dosing the lung have been completed. These studies essentially have all been Phase I safety studies that have delivered both viral and nonviral vectors topically to the nose and/or lower airways via direct liquid instillation or via aerosol. With respect to adenoviral vectors, both single and multiple dosing studies have been performed.

From these Phase I trials, there has been a wealth of data produced on the safety aspects of first-generation nonviral and viral vectors. In brief, there have been no instances of identification and/or recovery of recombinant viruses from viral vectors, and relatively few if any DNA/vector-specific systemic effects resulting from intrapulmonary vector instillation have been detected. There have been reports of both inflammatory adverse events and immunologic responses to vectors. With respect to acute inflammatory responses, tachykinin-mediated neuroinflammatory responses in the nasal cavity in response to high-dose adenoviral vectors have been reported. A syndrome associated with acute pulmonary inflammation has also been reported (9). It is not clear what the etiology of this latter syndrome may be and whether it reflects, in part, deposition of vectors on alveolar versus airway surfaces, and/or the immune status of the patients. An acute, presumably cytokine-mediated response to liposome-mediated gene transfer in the lung has also been reported (E. Alton, personal communication). With respect to more delayed immunologic responses, rather small increases in adenoviral neutralizing antibody titers have been reported without an adverse clinical outcome (10). Although the data are more sparse, few or no inflammatory/immunologic responses have been reported with the AAV vectors.

With respect to gene-transfer efficiency/efficacy, perhaps the most quantitative data available are from studies that have dosed the nasal epithelium. For adenoviral vectors, initial reports from unblinded studies using nasal PD protocols that discriminated poorly between the CF versus normal phenotype indicated functional correction of CF epithelial Cl⁻ transport (11). Data from larger, placebo-controlled and blinded studies indicate
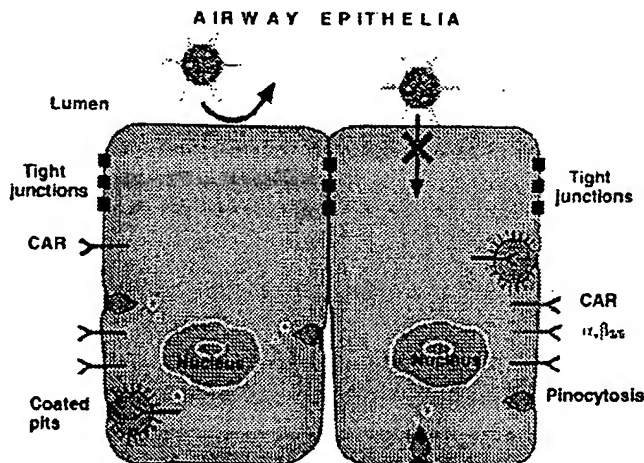
Figure 1

Barriers to vector-mediated gene transfer in WD columnar airway epithelial cells. The failure of vectors to bind to the apical membrane of WD cells is depicted on the left cell; the failure of "non-specifically" bound vectors to internalize is shown on the right cell. The tight junctions separate the apical cell membrane from the basolateral domain that selectively expresses specific viral receptors, e.g., the CAR, "housekeeping"/growth receptors, and integrins. WD, well-differentiated. CAR, Coxsackie virus and adenovirus receptor.

AIRWAY EPITHELIA

a
Lumen   A I R W A Y   E P I T H E L I A

Tight
Junctions

CAR

Coated
pits

CAR

Tight
Junctions

CAR
nucleus

Pinocytosis

b

bs-Ab

+● Agonist

7-TM

Tight
junctions

Tight
junctions

problem confronting CF gene therapy is the inefficiency of gene transfer. Whereas studies of inflammation and immunologic consequences of vector dosing are important and should be performed, such studies will not be fully informative until adequate gene transfer efficiency is achieved. This will allow the complex inflammatory/immunologic picture of expression of wild-type CFTR in the CF lung to be investigated properly.

Inefficient gene transfer reflects the extremely effective adaptations of airway epithelia to prevent the penetration of foreign materials into airway epithelial cells or the interstitium. Airway epithelia create a complex series of barriers to prevent penetration of lumenally delivered materials, including both viral and non-viral vectors, into the cell or interstitial compartment. In series, these barriers comprise a well-defined mucus layer that may bind inhaled vectors and clear them via mucus clearance mechanisms, a glycocalyx that may bind vectors and prevent binding to cell surface receptors, and perhaps most importantly, an apical cell membrane that is relatively devoid of viral receptors and growth/tropic receptors that internalize as part of their biology (Fig. 1). This series of barriers is complemented by epithelial tight junctions that are "moderately leaky" to ions but quite "tight" for larger solutes, thereby preventing penetration by current vectors from lumenal surfaces to the interstitium. Airway cells express most of the receptors that are used by current viral vectors for "virus-specific" entry on the basolateral membrane. Recent reports confirm that specific vector receptors, e.g., the adenovirus receptor (13), the AAV receptor (heparan sulfate) (14), and the VSV receptor (15) are indeed localized to the basolateral membrane. In addition, most of the housekeeping/growth/trophic hormone receptors are also located basolaterally.

The early studies with model systems that employed poorly differentiated airway epithelial cells suggested that gene-transfer efficiency for a variety of vectors

that topical delivery of adenoviral vector to the nasal epithelium results in little gene transfer or functional correction, as measured with a combination of molecular (PCR) and functional (nasal PD) techniques (12). Similarly, there is little evidence of significant gene transfer with liposome-mediated gene delivery in the nasal cavity, using a variety of lipids and plasmid systems. The data with AAV in the nose are preliminary but also suggest poor efficacy.

Efficacy studies in the lower airways are more difficult to perform because of the difficulty in defining the precise sites of vector delivery and the inability to assess gene transfer quantitatively. With respect to adenoviral gene transfer, PCR assessment of gene transfer has detected wild-type CFTR transcripts in brushings from dosed CF airways but there are few quantitative data measuring the percent transduced epithelial cells in the region and no functional (PD) measurements of correction. With respect to liposomes, one nicely designed and performed study attempted to measure functional and molecular correlates of CFTR expression after aerosolized liposome-plasmid dosing of the lung. These investigators reported perplexing evidence for modest correction of Cl- transport, but not Na⁺ transport function in the lung, without molecular (PCR) evidence of gene transfer (E. Alton, personal communication). Finally, although data from AAV administration to the lung are preliminary, they appear to show inefficiency as well.

What is the barrier to successful gene delivery? The major

would be high. However, with the advent of the use of well-differentiated (WD) culture systems, supplemented by freshly excised organ culture systems, it became clear that a common theme was emerging: a) that virtually all vectors (viral and nonviral) did not bind to the apical (lumenal) surfaces of WD airway epithelial cells; and b) that apical surfaces of WD airway epithelial cells have a low basal and stimulated rate of endocytosis (13, 16).

What is the answer to increase efficiency? It is apparent that novel strategies must be adopted to increase gene-transfer delivery. As mentioned above, strategies that may use the vascular compartment as a dosing route should be explored but the difficulties in overcoming the large number of barriers between the vascular compartment and airway epithelial cells — endothelial cell, endothelial cell basement membrane, interstitium, and epithelial basement membrane — make this route challenging. With respect to intralumenal dosing of the superficial epithelium, at least two general strategies can be envisioned to increase gene-transfer efficiency.

In a strategy termed "modification of the host," it may be rational to reduce the barrier functions of epithelial tight junctions so that vectors can penetrate to the basolateral membrane of target cells that, as indicated above, are naturally rich in viral and other internalizing receptors (Fig. 2). Abrogation of tight junction barrier function can be achieved by non-specific damage, such as has been demonstrated with oxidant gases (17) and surface-active adjuvants, e.g., detergents (18). Such strategies have been shown to increase gene-transfer efficiency in airways of rodents dramatically, but it likely will be difficult to titrate down the dose of an oxidant gas and/or deliver a specific mass of a detergent safely to make this strategy therapeutic for CF patients. More specific modifications of tight junctional permeability through cellular regulatory mechanisms thus are more appealing. Increasing knowledge of the cellular regulation of tight junctional permeabilities, including the interrelationships between the adherens junction and the tight junction, may make this approach feasible. The ultimate goal is a safe and effective strategy, which depends on: a) transient, reversible permeabilization of tight junctions; and b) permeabilization of tight junctions without producing inflammation, hence avoiding vascular leak into the airway lumen and airways irritation.

The alternative approach is to "modify the vector." The concept here is to direct a vector to a "target" expressed on the apical cell membrane that has the capacity to both bind and internalize a vector. Identification of suitable targets in the airway has not been easy because of the paucity of expressed receptor/membrane proteins on airway epithelial surfaces that internalize as a function of their biology. However, there is a class of receptors that normally mediates acute airway epithelial cell responses to the lumenal environment, i.e., seven transmembrane receptors. Several members of this class are expressed on the lumen of human airway cells.

Perhaps the most attractive target from the point of view of the level and extent of expression in the airways is the extracellular ATP/UTP receptor, termed P2Y2-R. This receptor internalizes into the cell via clathrin coated pits upon agonist stimulation. Many viruses have evolved mechanisms and escape from clathrin coated vesicles via a

process termed endosomolysis. Preliminary studies in non-polarized cells have documented that either bi-specific monoclonal antibodies directed towards engineered epitopes into the external domain of P2Y2-R or modifications of the native ligand (BiotinUTP) to direct vectors to P2Y2-R can produce efficient gene transfer via this pathway (19). More importantly, vector internalization and gene transfer can also be achieved when P2Y2-R is expressed in the apical membrane of WD cells. Targeting through this approach offers several attractive features, including a wide versatility with respect to the targeting molecules themselves, e.g., antibodies, peptides, or modified ligands, and the ability to link the targeting molecule to a wide variety of vectors, including plasmids, adenoviral, AAV, and lentiviral vectors.

Can we select a preferred vector now? This would be premature. A nonviral vector might be preferable because of the simplicity of the system, but a viral vector, if it were sufficiently safe and efficient, could be a viable alternative. It is likely that we will see a series of both types of vectors developed and used clinically. For example, it is possible that host modification or vector lumenal targeting will become a reality relatively rapidly, and that "high-capacity" adenoviral vectors, because of their proven ability to express in airway epithelial cells, their relative safety, and the transient nature of their expression, would be optimal for new studies of safety and efficacy. In the long term, it would appear reasonable that for a genetic disease like CF, integrative gene transfer will be preferable. Thus, it appears wise to continue the development of lentiviral vectors, both HIV (20) and non-human (21), and AAV vectors for this use.

The future. It is clear from analysis of the data describing gene-transfer efficiency from the reported clinical studies that an order or two of magnitude increase in efficiency will be required for gene transfer to be therapeutically relevant in CF. The good news is that all of the previous work has in principle identified the hurdles that must be cleared prior to initiation of novel strategies in man. For example, the WD cultures, freshly excised explant cultures, and bioelectric and expression studies in the mouse nose (but not infection phenotype in the lung) appear to be accurate models for predicting efficacy in man *in vivo*. Further, although there have been questions about its relevance, it does appear that the nose as a first approximation is a good model for lower airways gene transfer in man. Thus, the trial designs in the nose that have been generally agreed upon, i.e., double-blind placebo-controlled studies using nasal PD protocols designed to measure basal Na$^+$ transport and Cl$^-$ transport, coupled to molecular and morphologic studies with a spectrum of sensitivities, appear to offer a rigorous way to assess the efficacy of a new strategy before initiating more difficult studies in the lung.

A challenge for lung gene transfer, like other forms of CF lung therapy, will be the initial trial design to measure efficacy. Here again, much progress has been made. In the context of exploring drug therapy to treat the initiating cause of disease, trial designs have been explored to assess the ability of novel therapies to protect the lung against disease. Important analyses of the required sample sizes for these studies as a function of patient age have also been recently reported, and healthy discussions on surrogate markers in the lung

are ongoing (22). Thus, one can be optimistic that when we develop strategies that promote routinely between 10% and 100% gene-transfer efficiency in human airways, we will be smart enough not to miss the clinical benefits of gene transfer in CF patients.

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EXHIBIT 12

(12) **United States Patent**

Fisher

(10) Patent No.: **US 6,355,622 B1**

(45) **Date of Patent:** *Mar. 12, 2002

(54) **USE OF A MELANOMA DIFFERENTIATION ASSOCIATED GENE (MDA-7) FOR INDUCING APOPTOSIS OF A TUMOR CELL**

(75) Inventor: **Paul B. Fisher**, Scarsdale, NY (US)

(73) Assignee: **The Trustees of Columbia University in the City of New York**, New York, NY (US)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/251,124**

(22) Filed: **Feb. 16, 1999**

Related U.S. Application Data

(63) Continuation of application No. PCT/US97/14548, filed on Aug. 15, 1997, which is a continuation-in-part of application No. 08/696,573, filed on Aug. 16, 1996, now Pat. No. 5,710,137.

(51) Int. Cl.^{7} A01N 43/04; C12Q 1/68; C12P 21/06

(52) U.S. Cl. 514/44; 435/6; 435/69.1

(58) Field of Search 435/6, 69.1; 514/44

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Primary Examiner—Ardin H. Marschel

(57) **ABSTRACT**

This invention provides a method for reversing the cancerous phenotype of a cancer cell by introducing a nucleic acid having the melanoma differentiation associated gene (mda-7) into the cell under conditions that permit the expression of the gene so as to thereby reverse the cancerous phenotype of the cell. This invention also provides a method for reversing the cancerous phenotype of a cancer cell by introducing the gene product of the above-described gene into the cancerous cell so as to thereby reverse the cancerous phenotype of the cell. This invention also provides a pharmaceutical composition having an amount of a nucleic acid having the melanoma differentiation associated gene (mda-7) or the gene product of a melanoma differentiation associated gene (mda-7) effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier.

25 Claims, 6 Drawing Sheets

GROWTH OF HeLa CELLS TREATED WITH Ad.mda-7 S

NULL

Ad.mda-7 S
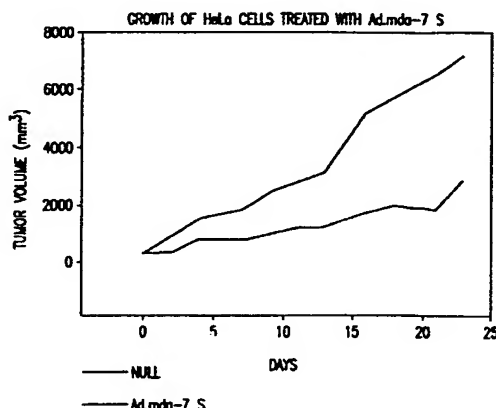
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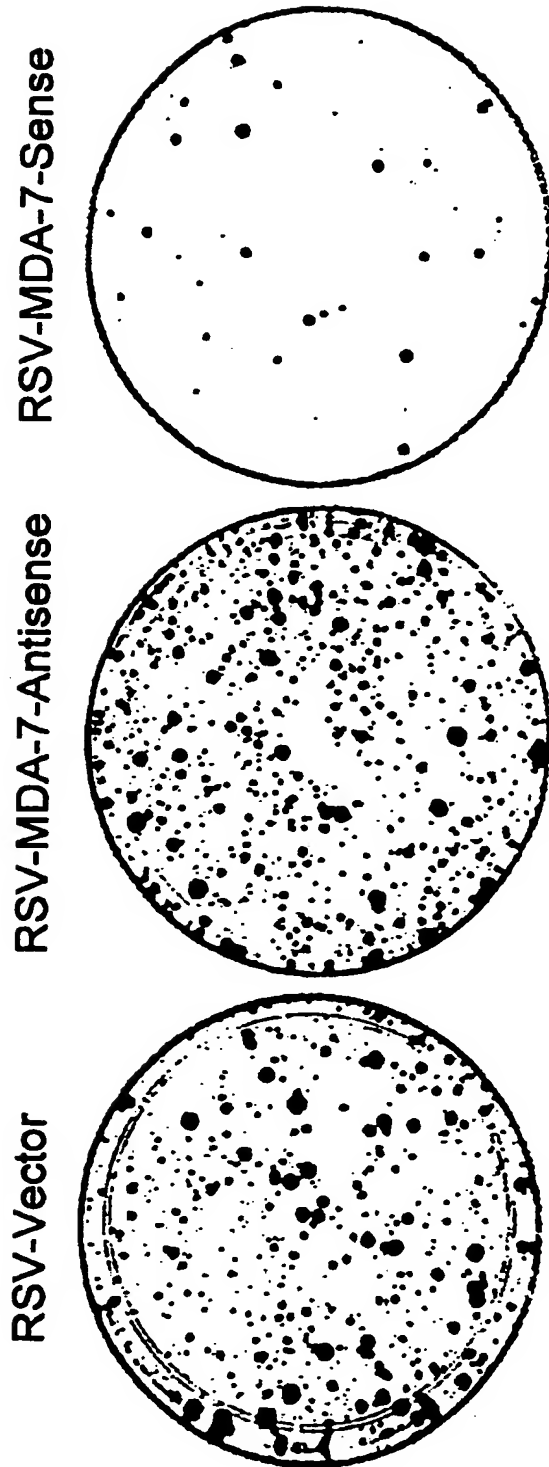
FIG.1
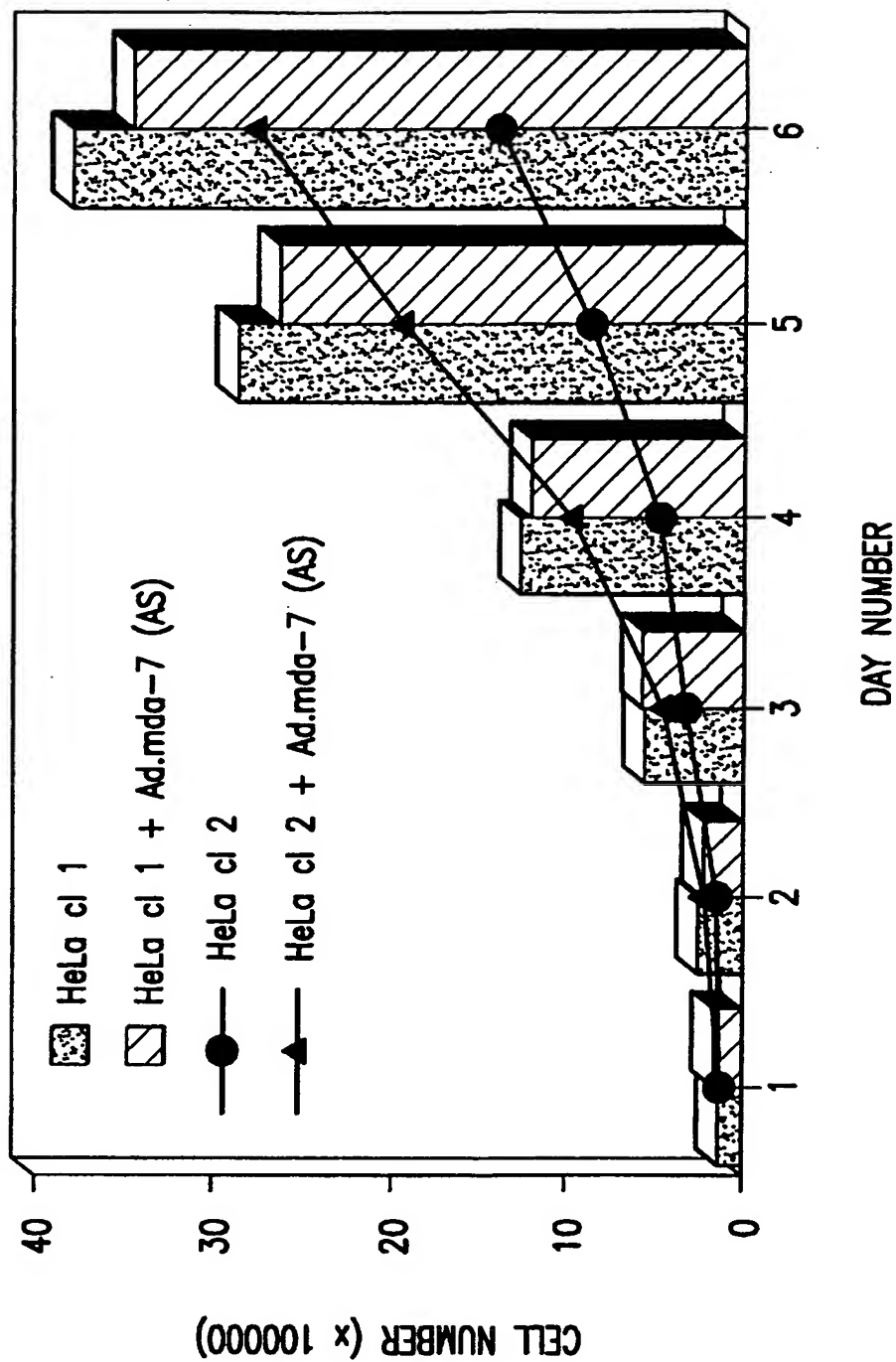
FIG.2
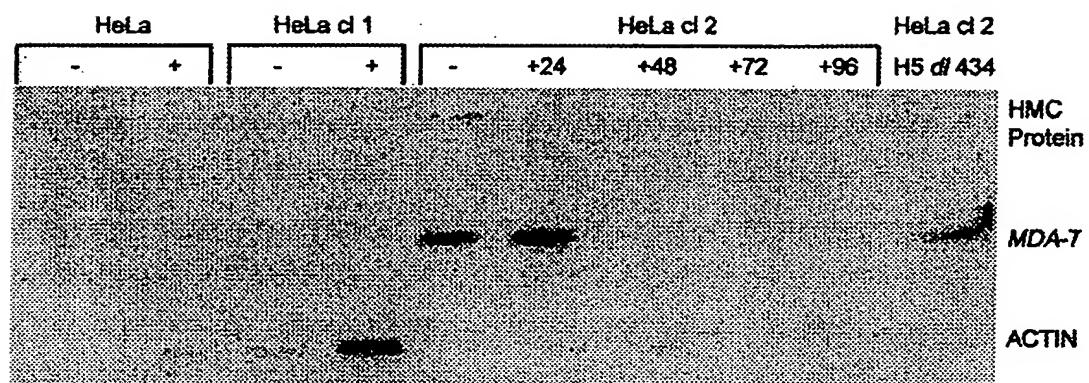
FIG.3
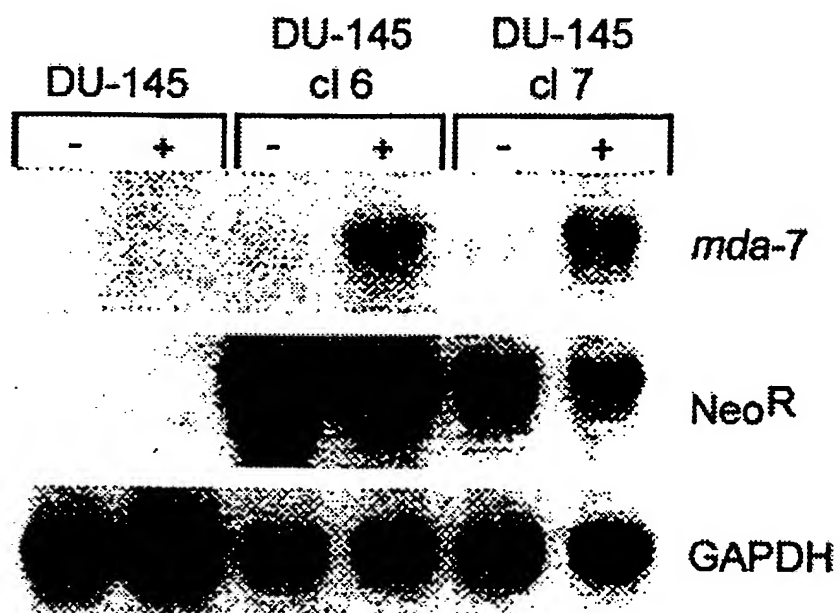
FIG.4A
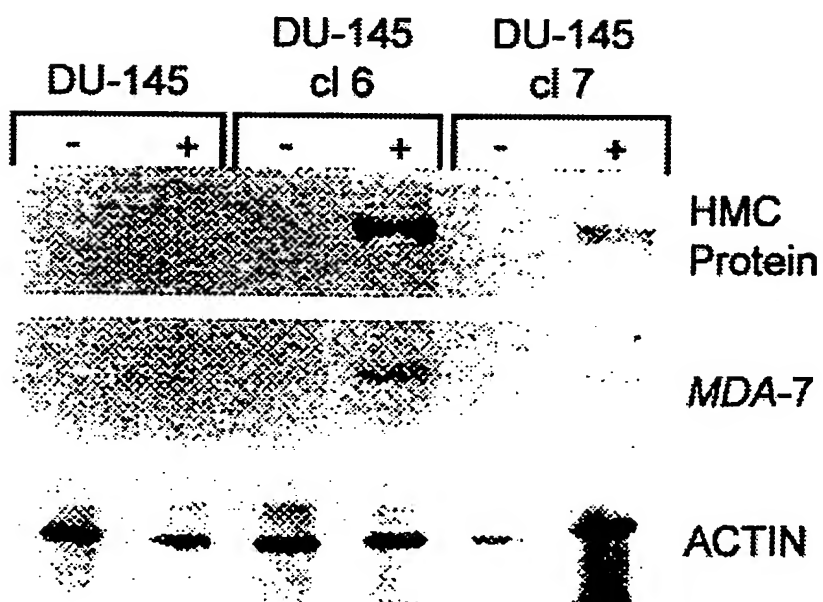
FIG.4B
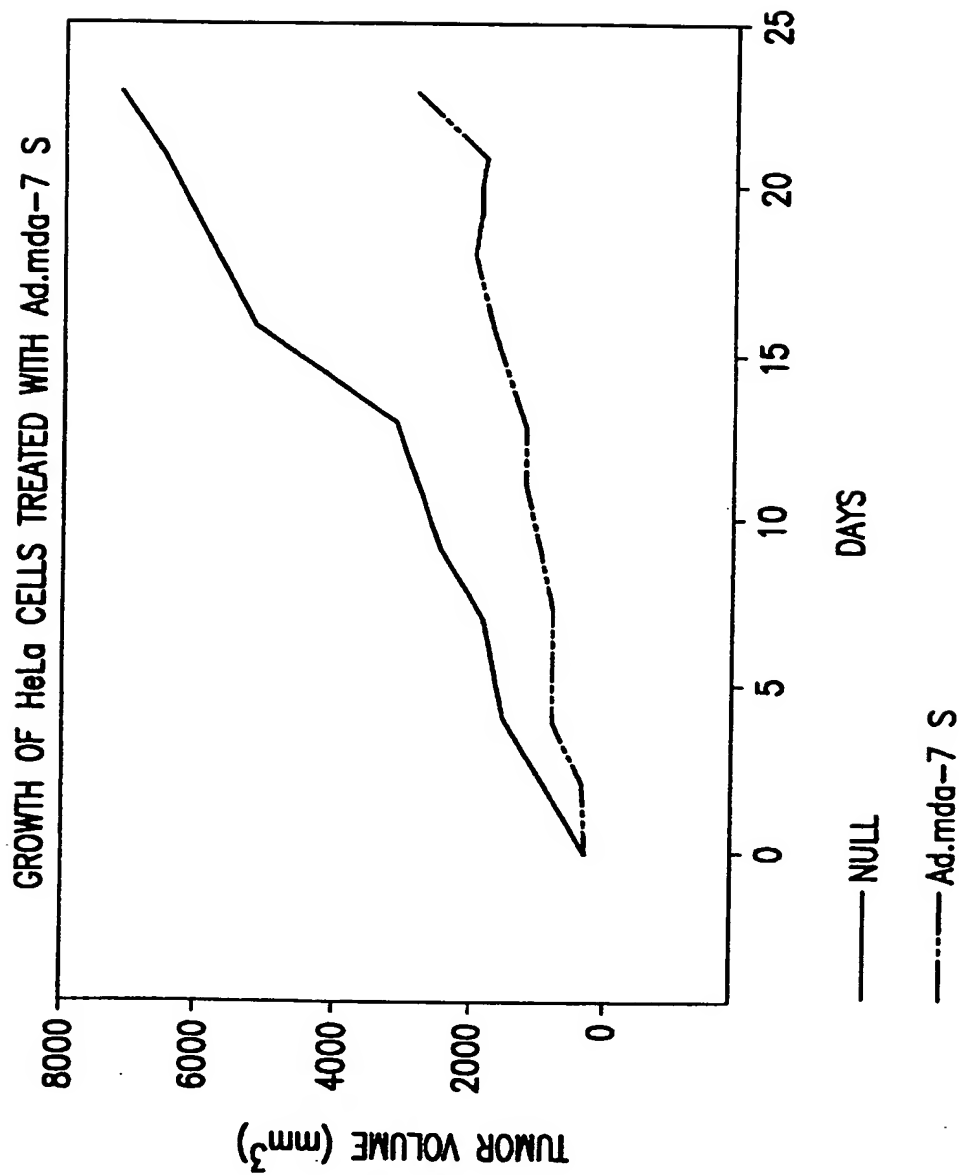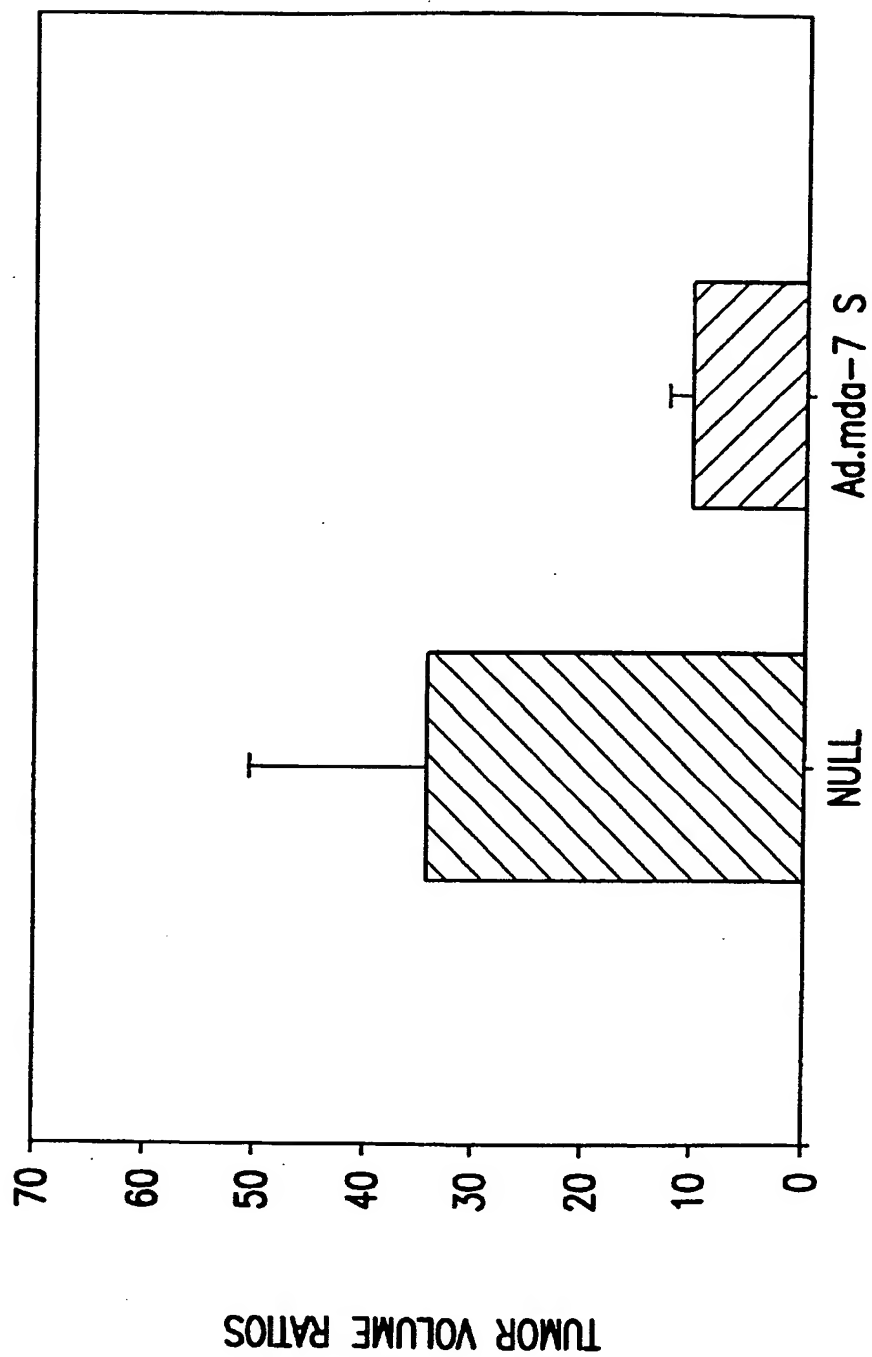
GROWTH OF HeLa CELLS TREATED WITH Ad.mda-7 S

FIG.5

FIG.6

USE OF A MELANOMA DIFFERENTIATION ASSOCIATED GENE (MDA-7) FOR INDUCING APOPTOSIS OF A TUMOR CELL

This application is a continuation of PCT International Application No. PCT/US97/14548, filed Aug. 15, 1997, designating the United States of America, which is a continuation-in-part of U.S. Ser. No. 08/696,573, filed Aug. 16, 1996, now U.S. Pat. No. 5,710,137; the contents of which are incorporated in their entireties into the present application.

The invention disclosed herein was made with Government support under NCI/NIH Grant No. CA35675 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of each series of experiments.

BACKGROUND OF THE INVENTION

Cancer is a complex multifactor and multistep process involving the coordinated expression and suppression of genes functioning as positive and negative regulators of oncogenesis (1–5). Direct cloning strategies, based on transfer of a dominant transforming or tumorigenic phenotype, have identified positive acting oncogenes (6–9). In contrast, the detection and cloning of genes that suppress the cancer phenotype have proven more difficult and elusive (10–15). A direct approach for isolating genes directly involved in regulating growth and differentiation involves subtraction hybridization between cDNA libraries constructed from actively growing cancer cells and cDNA libraries from cancer cells induced to lose proliferative capacity irreversibly and terminally differentiate (13,14). This experimental strategy has been applied to human melanoma cells, induced to terminally differentiate by treatment with recombinant human interferon β (IFN-β) and mezerein (MEZ), resulting in the cloning of novel melanoma differentiation-associated (mda) genes not previously described in DNA data bases (13,14). A direct role for specific mda genes in mediating growth and cell cycle control is apparent by the identification and cloning of mda-6 (13–16), which is identical to the ubiquitous inhibitor of cyclin-dependent kinases p21 (17). The importance of p21 in growth control is well documented and this gene has been independently isolated, as WAF-1, CIP-1, and SDI-1, by a number of laboratories using different approaches (18–20). These studies indicate that specific genes associated with proliferative control are induced and may contribute to the processes of growth arrest and terminal differentiation in human cancer cells.

The mda-7 gene was cloned from a differentiation inducer (IFN-β plus MEZ)-treated human melanoma (HO-1) subtracted library (13,14). The full-length mda-7 cDNA is 1718 nucleotides, and the major open reading frame encodes a novel protein of 206 aa with an M_r of 23.8 kDa (21). Previous studies indicate that mda-7 is induced as a function of growth arrest and induction of terminal differentiation in human melanoma cells (14,21). mda-7 expression also inversely correlates with melanoma progression—i.e., actively growing normal human melanocytes express more mda-7 than metastatic human melanoma cells (21). Moreover, mda-7 is growth inhibitory toward human melanoma cells in transient transfection assays and in stable

transformed cells containing a dexamethasone (DEX)-inducible mda-7 gene (21). These studies indicate that mda-7 may contribute to the physiology of human melanocytes and melanomas, and this gene has growth suppressive properties when overexpressed in human melanoma cells.

The mda-7 gene was also described in the International Patent Cooperation Treaty Application No. PCT/US94/12160, international filing date, Oct. 24, 1994 with International Publication No. WO95/11986, the content of which is incorporated into this application by reference.

This invention reports that mda-7 is a potent growth suppressing gene in cancer cells of diverse origin, including breast, central nervous system, cervix, colon, prostate and connective tissue. An inhibition in colony formation occurs in cancer cells containing defects in their p53 and/or retinoblastoma (RB) genes or lacking p53 and RB expression. In contrast, expression of mda-7 in normal human mammary epithelial cells, human skin fibroblasts and rat embryo fibroblasts induces quantitatively less growth suppression than in cancer cells. When stably expressed in human cervical carcinoma (HeLa) and prostate carcinoma (DU-145) cells, mda-7 has a negative effect on growth and transformation-related properties. The effects of mda-7 on HeLa cells are reversible following abrogation of the MDA-7 protein by infection with a genetically modified Ad5 vector expressing an antisense mda-7 gene. These observations indicate that mda-7 is a novel growth suppressing gene with a wide range of inhibitory actions in human cancers manifesting different genetic defects.

SUMMARY OF THE INVENTION

This invention provides a method for reversing the cancerous phenotype of a cancer cell by introducing a nucleic acid including a melanoma differentiation associated gene (mda-7) into the cell under conditions permitting the expression of the gene so as to thereby reverse the cancerous phenotype of the cell. This invention also provides a method for reversing the cancerous phenotype of cancer cell in a subject by introducing the above-described nucleic acid into the subject's cancerous cell.

This invention also provides a method for reversing the cancerous phenotype of a cancer cell by introducing the gene product of a melanoma differentiation associated gene (mda-7) into the cancer cell so as to thereby reverse the cancerous phenotype of the cell. This invention also provides a method for reversing the cancerous phenotype of a cancer cell in a subject by introducing the above-described gene product into the subject's cancerous cell.

This invention also provides a pharmaceutical composition having an amount of a nucleic acid including a melanoma differentiation associated gene (mda-7) effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier. This invention also provides a pharmaceutical composition having an amount of the gene product of the above-described gene effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Effect of mda-7 expression on hygromycin resistant colony formation in HeLa cells. HeLa cells were transfected with 10 μg of pREP4 vector (RSV-vector), mda-7 cloned in an antisense orientation in the pREP4 vector (RSV-MDA-7-Antisense), or mda-7 cloned in a sense orientation in the pREP4 vector (RSV-MDA-7-Sense) and selected in media containing 100 μg of hygromycin.

FIG. 2. Effect of antisense mda-7 on monolayer growth of pREP4 vector HeLa cl 1 and mda-7 (S) expressing HeLa cl 2 cells. HeLa cl 1 (pREP4 vector transformed HeLa clone) and HeLa cl 2 (mda-7 expressing HeLa clone) cells were grown in the absence or following infection with 10 plaque forming units/cell with a recombinant type 5 adenovirus (Ad5) expressing antisense mda-7 [Ad.mda-7 (AS)]. Results are the average cell number from triplicate samples that varied by $\leq 10\%$.

FIG. 3. Effect of antisense mda-7 on the high molecular weight-MDA-7 complexing (HMC) protein, the MDA-7 protein and the actin protein in HeLa, HeLa cl 1, and HeLa cl 2 cells. HeLa and HeLa cl 1 (pREP4 vector transformed HeLa clone) were uninfected (−) or infected (+) with 10 plaque forming units/cell of Ad.mda-7 (AS) for 96 hr labeled with [^{35}S] methionine, and the levels of the HMC, MDA-7 and actin proteins were determined by immunoprecipitation analysis. For HeLa cl 2 (mda-7 expressing HeLa clone), the effect of infection with 10 plaque forming units/ml of Ad.mda-7 (AS) on protein levels was determined by immunoprecipitation analysis of [^{35}S] methionine labeled cell lysates after +24, +48, +72 and +96 hr. The effect of infection of HeLa cl 2 cells with the control mutant Ad5, H5dl 434, was determined by immunoprecipitation analysis of [^{35}S] methionine labeled cell lysates 96 hr after infection with 10 plaque forming units/cell.

FIGS. 4A & 4B Synthesis of mda-7 RNA and protein in DU-145 clones containing a DEX-inducible mda-7 gene.

FIG. 4A. Cells were grown in the absence or presence of 10^{-6} M DEX for 96 hr, and total RNA was isolated, subjected to Northern blotting and probed with mda-7, a neomycin resistance (Neo^R) gene and GAPDH.

FIG. 4B. Cells were grown in the absence or presence of 10^{-6} M DEX for 96 hr, cellular proteins were labeled with [^{35}S] methionine and immunoprecipitated with antibodies recognizing MDA-7 and actin proteins.

FIG. 5 Inhibition of growth of established human cervical cancer (HeLa) exenografts in athymic nude mice.

FIG. 6 Effect of Ad.mda-7 S on HeLa tumor volume ratios. The result indicates that Ad.mda-7 S can inhibit tumor progression in vivo in nude mice.

DETAILED DESCRIPTION OF THE INVENTION

In order to facilitate an understanding of the Experimental Details section which follows, certain frequently occurring methods and/or terms are described in Sambrook, et al. (45).

This invention provides a method for reversing the cancerous phenotype of a cancer cell which comprises introducing a nucleic acid comprising a melanoma differentiation associated gene (mda-7) into the cell under conditions permitting the expression of the gene so as to thereby reverse the cancerous phenotype of the cell.

This invention also provides a method for reversing the cancerous phenotype of a cancer cell in a subject which comprises introducing a nucleic acid molecule comprising a melanoma differentiation associated gene (mda-7) into the subject's cancerous cell under conditions permitting expression of the gene in the subject's cells so as to thereby reverse the cancerous phenotype of the cell.

Methods to introduce a nucleic acid molecule into cells have been well known in the art. Naked nucleic acid molecule may be introduced into the cell by direct transformation. Alternatively, the nucleic acid molecule may be embedded in liposomes. Accordingly, this invention pro-

vides the above methods wherein the nucleic acid is introduced into the cells by naked DNA technology, adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV vector, retroviral vectors, vaccinia virus vector, liposomes, antibody-coated liposomes, mechanical or electrical means. The above recited methods are merely served as examples for feasible means of introduction of the nucleic acid into cells. Other methods known may be also be used in this invention.

In an embodiment of the above methods, the melanoma differentiation associated gene (mda-7) is linked to a regulatory element such that its expression is under the control of the regulatory element. In a still further embodiment, the regulatory element is inducible or constitutive. Inducible regulatory element like an inducible promoter is known in the art. Regulatory element such as promoter which can direct constitutive expression is also known in the art.

In a separate embodiment, the regulatory element is a tissue specific regulatory element. The expression of the mda-7 gene will then be tissue-specific.

In another embodiment of the above-described methods, the cancer cell is characterized by the presence within the cancer cell of a defective tumor suppressor gene. The defective tumor suppressor gene includes, but is not limited to, a p53, a retinoblastoma (RB) or a p16^{ink4a} gene.

In an embodiment of the above-described methods, the cancer cell is characterized by the presence within the cancer cell of a dominant acting oncogene. Specifically, the dominant acting oncogene may be a Ha-ras, mutant p53 or human papilloma virus genes. The Ha-ras is a Harvey virus ras oncogene.

In an embodiment of the above methods, the nucleic acid comprises a vector. The vector includes, but is not limited to, an adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV vector, retrovirus vector and vaccinia virus vector. In a preferred embodiment, the adenovirus vector is a replication-defective adenovirus vector expressing mda-7, designated Ad.mda-7 S. In another embodiment, the adenovirus vector is a replication-competent adenovirus vector.

This invention also provides a method for reversing the cancerous phenotype of a cancer cell which comprises introducing the gene product of a melanoma differentiation associated gene (mda-7) into the cancerous cell so as to thereby reverse the cancerous phenotype of the cell.

This invention further provides a method for reversing the cancerous phenotype of a cancer cell in a subject which comprises introducing the gene product of a melanoma differentiation associated gene (mda-7) into the subject's cancerous cell so as to thereby reverse the cancerous phenotype of the cell.

In an embodiment of the above-described methods, the cancer cell includes, but is not limited to, a breast, cervical, colon, prostate, nasopharyngeal, lung connective tissue or nervous system cell. The cancer cell further includes cells from glioblastoma multiforme, lymphomas and leukemia.

This invention also provides a pharmaceutical composition which comprises an amount of a nucleic acid comprising a melanoma differentiation associated gene (mda-7) effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers. The pharmaceutical composition may be constituted into any form suitable for the mode of administration

selected. Compositions suitable for oral administration include solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

In an embodiment, the nucleic acid comprises a vector. The vector includes, but is not limited to, an adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV virus, retrovirus vector and vaccinia virus vector. In a preferred embodiment, the adenovirus vector is a replication-defective adenovirus vector expressing mda-7, designated Ad.mda-7 S. In another embodiment, the adenovirus is a replication-competent adenovirus vector.

This invention also provides a pharmaceutical composition comprising an amount of the gene product of a melanoma differentiation associated gene (mda-7) effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier.

In an embodiment of the above-described methods, the cancer cell includes, but is not limited to, a breast, cervical, colon, prostate, nasopharyngeal, lung connective tissue and nervous system cells. The cancer cell further includes cells from glioblastoma multiforme, lymphomas and leukemia.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Cancer is a disease characterized by defects in growth control, and tumor cells often display abnormal patterns of cellular differentiation. The combination of recombinant human fibroblast interferon and the antileukemic agent mezerein corrects these abnormalities in cultured human melanoma cells resulting in irreversible growth arrest and terminal differentiation. Subtraction hybridization identifies a melanoma differentiation associated gene (mda-7) with elevated expression in growth arrested and terminally differentiated human melanoma cells. Colony formation decreases when mda-7 is transfected into human tumor cells of diverse origin and with multiple genetic defects. In contrast, the effects of mda-7 on growth and colony formation in transient transfection assays with normal cells, including human mammary epithelial, human skin fibroblast and rat embryo fibroblast, is quantitatively less than that found with cancer cells. Tumor cells expressing elevated mda-7 display suppression in monolayer growth and anchorage independence. Infection with a recombinant type 5 adenovirus expressing antisense mda-7 eliminates mda-7 suppression of the in vitro growth and transformed phenotype. The ability of mda-7 to suppress growth in cancer cells not expressing or containing defects in both the retinoblastoma (RB) and p53 genes indicates a lack of involvement of these critical tumor suppressor elements in mediating mda-7-induced growth inhibition. The lack of protein homology of mda-7 with previously described growth suppressing genes and the differential effect of this gene on normal versus cancer cells suggests that mda-7 may represent a new class of cancer growth suppressing genes with antitumor activity.

Materials and Methods

Cell Lines and Culture Conditions. Human carcinoma cell lines, including MCF-7 and T47D (breast), LS174T and SW480 (colorectal), HeLa (cervical), DU-145 (prostate), and HONE-1 (nasopharyngeal) (9,22–25), were grown in

Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM-10) at 37° C. in a 5% CO_2/95% air-humidified incubator. Additional human cell types including HBL-100 (normal mammary epithelial), HO-1 and C8161 (melanoma), GBM-18 and T98G (glioblastoma multiforme) and Saos-2 (human osteosarcoma) were maintained under similar conditions. Early passage normal human mammary epithelial cells (HMEC; passages 10–12) were obtained from Clonetics Corporation (San Diego, Calif.) HMEC cells were maintained in serum-free medium as described by Clonetics Corporation. CREF-Trans 6 (cloned Fischer rat embryo fibroblast) (9,26) and CREF Ha-ras (CREF cells transformed by the Ha-ras (T24) oncogene) (27) were cultured in DMEM-5. HeLa cl 1 is a hygromycin resistant (Hyg^R) Rous Sacroma virus RSV vector (pREP4) (Invitrogen) transformed HeLa clone. HeLa cl 2 is a Hyg^R mda-7 expressing HeLa clone. HeLa cl 1 and HeLa cl 2 cells were constructed as described (12,21) and maintained in DMEM-10 containing 100 μg/ml of hygromycin. DU-145 cl 6 and DU-145 cl 7 cells contain a DEX-inducible mda-7 gene (cloned in a pMAMneo vector) (Clontech) (21) and are maintained in DMEM-10 containing 200 μg/ml G418.

Subtraction Hybridization, Plasmids, Expression Vector Constructs, and Northern Hybridization. Identification and cloning of mda-7 by subtraction hybridization was achieved as described (13). A full-length mda-7 cDNA was isolated by screening a recombinant IFN-β plus MEZ-treated HO-1 cDNA library (13) and using the procedure of rapid amplification of cDNA ends as described (15). An mda-7 cDNA fragment (nucleotide position 176–960) containing the open reading frame was amplified with PCR and cloned into pCRII™ (Invitrogen) by TA cloning. The orientation of the inserts in the vectors was determined by restriction mapping. The human cell expression constructs were made by cloning Kpn I-Xho I fragments from the PCR™ vectors into pREP4 vector (Invitrogen) downstream of a RSV promoter in a sense [mda-7 (S)] or antisense [mda-7 (AS)] orientation. Alternatively, the mda-7 gene fragment was cloned into the pMAMneo (Clontech) vector in a sense and antisense orientation. RNA isolation and Northern blotting were performed as described (9,12,13,21).

Monolayer Growth, Anchorage-Independence and DNA-Transfection Assays. Monolayer and anchorage-independent growth assays were performed as previously described (8,12,26). To study the effect of mda-7 on monolayer colony formation the vector [pREP4 (RSV)] containing no insert, mda-7 (S) or mda-7 (AS) expression constructs were transfected into the various cell types by the lipofectin method (GIBCO/BRL) and hygromycin resistant colony formation or cell growth in hygromycin was determined (12,21).

Construction of Antisense-mda-7 Adenovirus Vector. The recombinant replication-defective Ad.imda-7 (AS) was created in two steps. First, the coding sequence of the mda-7 gene was cloned into a modified Ad expression vector pAd.CMV (28). This contains, in order, the first 355 bp from the left end of the Ad genome, the cytomegalovirus (CMV) immediate early promoter, DNA encoding splice donor and acceptor sites, cloning sites for the desired gene (in this case mda-7), DNA encoding a polyA signal sequence from the beta globin gene, and approximately 3 kbp of adenovirus sequence extending from within the E1B coding region. This arrangement allows high level expression of the cloned sequence by the CMV immediate early gene promoter, and appropriate RNA processing (28). The recombinant virus was created in vivo in 293 cells (29) by homologous

recombination between mda-7-containing vector and plasmid JM17, which contains the whole of the Ad genome cloned into a modified version of pBR322 (30). JM17 gives rise to Ad genomes in vivo but they are too large to package. This constraint is relieved by recombination with the vector to create a packageable genome (30), containing the gene of choice. The recombinant virus is replication defective in human cells except 293 cells, which express adenovirus E1A and E1B. Following transfection of the two plasmids, infectious virus was recovered, the genomes were analyzed to confirm the recombinant structure, and then virus was plaque purified, all by standard procedures (31).

Peptide Antibody Production and Immunoprecipitation Analyses. Peptide antibodies were prepared against PSQEN-EMFSIRD (SEQ ID NO.: 1) as described (21). Logarithmically growing HeLa, HeLa cl 1 (Hyg^R pREP4 vector control HeLa clone), and HeLa cl 2 [pREP4-mda-7 (S) transfected Hyg^R mda-7 expressing HeLa clone] cells were either untreated or infected with 10 plaque forming units of control adenovirus (H5dl434) (32) or a recombinant adenovirus expressing mda-7 (AS) [Ad.mda-7 (AS)]. At various times after infection, cultures were starved of methionine for 1 hr at 37° C. in methionine-free medium, cells were concentrated by pelleting and labeled for 4 hr at 37° C. in 1 ml of the same medium with 100 μCi (1 Ci=37GBq) of ^{35}S (NEN; Express ^{35}S). Immunoprecipitation analyses with 2 μg of MDA-7 peptide rabbit polyclonal antibody or actin monoclonal antibody (Oncogene Sciences) were performed as described (15,21).

Experimental Results

Enhanced Growth Inhibitory Properties of mda-7 in Human Cancer Cells and Ha-ras-Transformed Rat Embryo Fibroblast Cells. DNA transfection assays were performed to evaluate the effect of elevated expression of mda-7 on cell growth. When transfected into human cervical carcinoma (HeLa) cells, the mda-7 (S) construct results in a 10- to 15-fold reduction in Hyg^R colonies in comparison with the pREP4 vector and mda-7 (AS) construct transfected cultures (FIG. 1 and Table 1).

TABLE 1

Effect of mda-7 on monolayer colony formation of human cancer, normal rat embryo fibroblast (CREF) and Ha-ras-transformed CREF cells.

Cell Type	RSV-Vector[a]	RSV-mda-7 (S)[b]	RSV-mda-7 (AS)
Human cancer cell lines[c]			
MCF-7 (Breast-Ca)	118 ± 24	42 ± 16 (3.5)	146 ± 20
T47D (Breast-Ca)	172 ± 9	44 ± 7 (4.2)	186 ± 28
HeLa (Cervix-Ca)	1571 ± 446	117 ± 107 (15.2)	1771 ± 385
LS174T (Colorectal-Ca)	130 ± 14	30 ± 3 (5.4)	160 ± 15
HONE-1 (Naso-pharyngeal-Ca)	219 ± 19	71 ± 8 (3.5)	250 ± 19
DU-145 (Prostate-Ca)	174 ± 18	54 ± 8 (3.1)	166 ± 12
T98G (Glioblastoma)	99 ± 9	32 ± 4 (3.6)	115 ± 14
Saos-2 (Osteosarcoma)	126 ± 22	35 ± 6 (3.9)	138 ± 14

TABLE 1-continued

Effect of mda-7 on monolayer colony formation of human cancer, normal rat embryo fibroblast (CREF) and Ha-ras-transformed CREF cells.

Cell Type	RSV-Vector[a]	RSV-mda-7 (S)[b]	RSV-mda-7 (AS)
Rat embryo fibroblast			
CREF (normal rat embryo)	60 ± 10	35 ± 5 (1.7)	66 ± 7
CREF-ras (transformed)	147 ± 16	25 ± 4 (6.0)	151 ± 16

[a]Logarithmically growing cells were seeded at 1 x 10^6 per 100 mm plate and transfected with 10 μg of vector [pREP4 (RSV)] containing no insert, mda-7 (S), or mda-7 (AS). After 24 hr, cells were replated at approximately 2 x 10^5 cells per 100 mm plate in medium containing 100 μg/ml of hygromycin. Medium was changed every 3 or 4 days and plates were fixed in formaldehyde and stained with Giemsa at day 14 or 21. Colonies containing 50 or more cells were enumerated. Values shown are the average Hyg^R colonies formed in four to five replicate plates ± S.D.
[b]Values in parentheses indicate fold-decrease in colony formation versus RSV-mda-7 (AS) transfected cells.
[c]MCF-7, T47D, HeLa, LS174T, DU-145 and HONE-1 are human carcinoma (Ca) cell lines isolated from the indicated anatomical site. T98G is a human glioblastoma multiforme cell line. CREF-ras is a Ha-ras (T24) oncogene transformed CREF clone.

In addition to forming fewer colonies, mda-7 (S) colonies are generally smaller in size than corresponding Hyg^R colonies resulting after transfection with the pREP4 vector or mda-7 (AS) constructs (FIG. 1). When transfected into additional human cancer cell lines mda-7 (S) constructs reduce Hyg^R colony formation by 3- to 10-fold (Table 1). These include human breast carcinoma (MCF-7 and T47D), colon carcinoma (LS174T and SW480), nasopharyngeal carcinoma (HONE-1), prostate carcinoma (DU-145), melanoma (HO-1 and C8161), glioblastoma multiforme (GBM-18 and T98G) and osteosarcoma (Saos-2). As observed with HeLa cells, the average sizes of Hyg^R colonies that form after transfection with mda-7 (S) constructs are smaller than those formed following transfection with the empty pREP4 vector or mda-7 (AS) constructs. These results demonstrate that mda-7 is a potent growth suppressing gene when over-expressed in a wide spectrum of histologically distinct human cancers.

To determine if mda-7 also inhibits the growth of normal cells and whether this effect is quantitatively similar to that observed with human cancer cells, transient DNA transfection assays were performed with passage 10 to 12 normal human mammary epithelial (HMEC) cells, the normal breast epithelial cell line HBL-100, normal human skin fibroblasts (passage 21) and a cloned normal rat embryo fibroblast cell line (CREF-Trans 6) (7,8). Since HMEC, HBL-100 and normal human skin fibroblasts do not form well-defined colonies at high frequencies, even when using a feeder-layer, the effect on total cell number after transfection with the different RSV constructs and growth for two and three weeks in hygromycin was determined. Using this approach, an approximate 1.1 to 1.6-fold decrease in HMEC, an approximate 1.1 to 1.2-fold decrease in HBL-100 and an approximate 1.3 to 2.1-fold decrease in normal human skin fibroblast cell number was observed (three independent experiments with each cell type) in mda-7 (S) versus mda-7 (AS) or pREP4 vector transfected normal cells, respectively. In contrast, using a similar experimental protocol with T47D human breast carcinoma cells, growth was inhibited following transfection with the mda-7 (S) construct approximately 3.2 to 5.2-fold in comparison with vector-and antisense-

transfected cells. In the case of CREF-Trans 6 cells, the difference in Hyg^R colony formation for six independent transfection assays between mda-7 (S) versus mda-7 (AS) and vector transfected cells ranged from 0.5 to 2.8-fold (Table 1). In contrast, transfection of mda-7 (S) constructs into Ha-ras transformed CREF cells reduced colony formation by ~6 to 8-fold (Table 1). These results indicate that mda-7 is quantitatively less effective in reducing growth and colony formation in normal human and normal rodent cells than in human cancer and Ha-ras-transformed rat embryo cells.

Effect of Stable and Inducible mda-7 Expression and Antisense Inhibition of mda-7 Expression on Cell Growth and the Transformed Phenotype. To determine the reason for low frequency HeLa cell survival after transfection with the mda-7 (S) gene, ten independent Hyg^R colonies were isolated following transfection with the mda-7 (S) construct. Of the 10 clones analyzed by Northern blotting for mda-7 expression, 7 clones did not express detectable mda-7 mRNA, 2 clones expressed low levels of mda-7 mRNA and 1 clone (designated HeLa cl 2) displayed high levels of mda-7 mRNA. In contrast, all of the clones displayed comparable levels of Hyg^R and glyceraldehyde 3-phsphate dehydrogenase (GAPDH) gene expression. When compared with parental HeLa cells or an pREP4 vector HeLa clone (designated HeLa cl 1), HeLa cl 2 (mda-7 expressing) cells grew at a reduced rate (FIG. 2). When grown in agar, uncloned HeLa and HeLa cl 1 cells grew with approximately 42% efficiency, whereas HeLa cl 2 (mda-7 expressing) cells grew with approximately 25% efficiency and the average sizes of colonies were smaller than observed with parental HeLa and pREP4 vector HeLa cl 1 cells. These results indicate that HeLa survival after transfection with mda-7 results primarily from the lack of or low levels of mda-7 expression. However, in HeLa cells that stably express elevated mda-7, growth in monolayer culture and anchorage-independence are reduced.

To determine if the reduction in in vitro growth and transformation suppression found in HeLa cl 2 (mda-7 expressing) are a direct consequence of mda-7 expression, an antisense strategy was used to directly inhibit mda-7 expression. A recombinant Ad5 vector containing the mda-7 gene cloned in an antisense orientation [Ad.mda-7 (AS)] was constructed. Infection of HeLa cl 2 (mda-7 expressing), but not HeLa cl 1 (pREP4 vector, non-mda-7 expressing) or parental HeLa, with Ad.mda-7 (AS) increases growth rate and agar cloning efficiency (from approximately 25 to approximately 44%) (FIG. 2). In contrast, the control mutant Ad5 vector (H5dl434), not containing the mda-7 gene, does not affect monolayer or agar growth of parental HeLa, HeLa cl 1 or HeLa cl 2 cells (data not shown).

Using mda-7-specific peptide antibodies produced in rabbits and immunoprecipitation analyses, the HeLa cl 2 (mda-7 expressing) cells contain elevated levels of the MDA-7 approximately 24 kDa protein and a high molecular weight complexing (HMC) protein of approximately 90 to 110 kDa (FIG. 3). Infection with Ad.mda-7 (AS), but not the H5dl434 control non-mda-7 expressing virus, results in a temporal decrease in both the ~24 kDa MDA-7 protein and the HMC protein (21) (FIG. 3). Reduced levels of both proteins are seen by 48 hr and remain suppressed over a 96 hr period after infection with Ad.mda-7 (AS). In contrast, actin levels remain unaltered following viral infection. These findings indicate that antisense inhibition of MDA-7 protein expression in HeLa cl 2 (mda-7 expressing) can directly extinguish mda-7 induced growth suppression and inhibition in anchorage-independent growth.

To confirm the suppressive effect of mda-7 on cell growth, DU-145 human prostate cancer cells were engineered to express a DEX-inducible mda-7 gene. When DU-145 cl 6 or cl 7 cells [containing a DEX-inducible mda-7 (S) gene], but not parental DU-145 cells, are grown for 24 to 96 hr in the presence of 10^{-6} M DEX, mda-7 mRNA and protein (including the HMC protein) are induced (FIG. 4). In contrast, DEX does not alter neomycin resistance (Neo^R) gene expression in DU-145 cl 6 and cl 7 cells or GAPDH expression in any of the cells tested (FIG. 4). Induction of mda-7 expression in DU-145 cl 6 and cl 7 cells by growth in 10^{-6} M DEX results in approximately 50% reduction in cell number after 96 hr versus growth in the absence of DEX. In contrast, no significant growth inhibition occurs when parental DU-145 or pMAMneo vector transformed DU-145 cells are grown for 96 hr in medium containing 10^{-6} M DEX (data not shown). These data indicate that ectopic expression of mda-7 can directly alter cell growth in prostate cancer cells.

Experimental Discussion

Subtraction hybridization identified mda genes with elevated expression in growth arrested and terminally differentiated human melanoma cells (13,14,21). Determining the function of these mda genes will be paramount in defining the molecular basis of growth control and terminal differentiation in human melanoma and other cell types. The mda-7 gene (14,21) is now shown to be a ubiquitous growth suppressing gene when transiently or stably expressed in a wide array of human cancer cell lines. This finding extends previous observations indicating growth inhibitory properties of the MDA-7 protein in human melanoma cells (21). In contrast to its effects on cancer cells, transfection of mda-7 into normal human mammary epithelial, normal human skin fibroblast and normal rat embryo fibroblast cells results in quantitatively less growth suppression. Like another mda gene, mda-6 (p21), mda-7 expression is also inversely correlated with melanoma progression, with elevated levels of both mda-6 (p21) and mda-7 present in normal human melanocytes relative to metastatic human melanoma cells (14-16,21). Since normal melanocytes still retain proliferative capacity, although at a reduced rate relative to melanoma cells, it is possible that both mda-6 (p21) and mda-7 function as negative regulators of the progression phenotype in melanocyte/melanoma lineage cells (14-16,21). Moreover, the elevated expression of both mda-6 (p21) and mda-7 in terminally differentiated and irreversibly growth arrested human melanoma cells, suggests that these genes may also be important regulators of the terminal differentiation phenotype (13-16,21).

The mechanism by which mda-7 elicits its growth suppressive effects on human cancer cells is not presently known. The structure of mda-7 does not provide insight into potential function, since no sequence motifs are present that would suggest a potential mode of action. The effect of mda-7 on cell growth can be distinguished from the extensively studied tumor suppressor gene p53 (33,34). Transient expression of p53 in the mutant p53 containing T47D human breast carcinoma cell line results in growth suppression, whereas transfection of a wild-type p53 gene into the wild-type p53 containing MCF-7 human breast carcinoma cell line does not induce growth inhibition (34). In contrast, mda-7 induces similar growth suppression in both T47D and MCF-7 cells (Table 1). Growth inhibition by mda-7 can also be disassociated from that observed with the retinoblastoma gene (pRB), the pRb-associated p107 gene and the putative tumor suppressor gene p16^{ink4} (25,35). Overexpression of pRb and p107 inhibit cellular proliferation in specific cell

types and in a cell cycle-dependent manner (35–37). Transfection of pRb or p107 into the human glioblastoma cell line T98G that contains an apparently normal RB gene (25) does not induce growth suppression (35,37), whereas transient mda-7 (S) expression reduces T98G colony formation (Table 1). At the present time, the growth inhibitory effect of mda-7 cannot be distinguished from growth suppression induced by the RB family member p130/pRb2, which also inhibits proliferation in T98G cells (25). The p16^{ink4} gene induces growth arrest in cells containing a functional RB gene (35,37), whereas mda-7 growth suppression occurs in cells containing normal, abnormal or non-functional RB genes. Transfection of mda-7 into the DU-145 human prostate carcinoma cell line that contains a mutated RB gene (38) and Saos-2 human osteosarcoma cells that do not express RB (or wild-type p53) results in an inhibition in colony formation (Table 1). Similarly, induction of mda-7 expression in stable DEX-inducible mda-7 transformed DU-145 clones results in growth suppression. These findings indicate a lack of dependence on a functional RB gene for growth inhibition by mda-7. Taken together these studies demonstrate that the inhibitory effect of mda-7 occurs by a mechanism that is distinct from the mode of action of the two most extensively studied tumor suppressor genes, p53 and pRb, and the putative tumor suppressor gene p16^{ink4}.

Several genes have been identified that display elevated expression as a function of growth arrest or DNA damage in mammalian cells (39,40). Three growth arrest and DNA damage inducible (gadd) genes, gadd45, gadd153 and gadd34, the closely related myeloid differentiation primary response (MyD118) gene (41) and the wild-type p53 inhibiting gene mdm-2 (42) are upregulated in cells by treatment with the DNA damaging agent methyl methanesulfonate (MMS) (40). The gadd45 and growth arrest-specific gene (gas1) (43,44) are induced by maintaining cells at confluence, serum-starving cells or growing cells in low serum (40,43,44). In contrast, mda-7 mRNA expression is not induced in human melanoma cells following treatment with methyl methane sulfonate (MMS) or after maintaining cells at confluence (21). Moreover, only a small increase in mda-7 mRNA expression occurs in H0-1 human melanoma cells following growth in serum-free medium for 96 hr (21). The difference in regulation of mda-7 versus the gadd, MyD118 and gas-1 genes indicates that mda-7 may represent a new class of growth arresting genes.

In summary, a negative growth regulator, mda-7, is described that induces growth suppression in human cancer cells containing both normal and mutated p53 and RB genes. Characterization of the genomic structure of mda-7 will be important in determining if this gene normally functions as a tumor suppressor gene and whether alterations are present in this gene in tumor versus normal cells. Identification of the promoter region of mda-7 will also permit an analysis of the mechanism by which this gene is differentially expressed and inducible by IFN-β plus MEZ in specific cell types. Of potential importance and warranting expanded studies, is the finding that mda-7 is more growth inhibitory toward cancer and transformed cells than normal cells. In this context, mda-7 could prove useful as part of a gene-based interventional strategy for cancer therapy, in an analogous manner as the wild-type p53 gene is currently being tested for efficacy in the therapy of specific human malignancies.

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Second Series of Experiments

Melanoma Differentiation Associated Gene-7 (mda-7) in a Recombinant Adenovirus Inhibits the Growth of Established Human Tumors in Nude Mice

Previous studies document that ectopic expression of mda-7 in human tumor cells of diverse origins inhibits growth, as documented by a decrease in colony formation in monolayer culture (Jiang et al., PNAS, 93: 9160–9165, 1996). In contrast, mda-7 does not significantly alter the growth of normal human epithelial or fibroblast cells. These observations support the hypothesis that mda-7 is a ubiquitous cancer growth suppressor gene.

The ability of mda-7 to selectively inhibit cancer cell growth suggests that this gene might provide therapeutic benefits in the treatment of human cancers. To explore this possibility a replication-defective adenovirus expressing mda-7 has been generated. The protocols were similar to those used to construct an adenovirus expressing antisense mda-7, Ad.mda-7 AS (Jiang et al., PNAS, 93: 9160–9165, 1996). The recombinant replication-defective Ad.mda-7 S was produced in two steps. First, the mda-7 gene was cloned in a sense orientation into a modified Ad expression vector pAd.CMV. This virus contains, in order, the first 355 bp from the left end of the Ad genome, the cytomegalovirus (CMV) immediate early promoter, DNA encoding a poly A signal sequence from the beta globin gene, and approximately 3 kbp of adenovirus sequence extending from within the E1B coding region. This arrangement allows high level expression of the cloned sequence by the CMV immediate early gene promoter, and appropriate RNA processing. The recombinant virus was created in vivo in 293 cells by homologous recombination between mda-7 containing vector and JM17, which contains the whole of the Ad genome cloned into a modified version of pBR322. JM17 gives rise to Ad genomes in vivo but they are too large to package. This

constraint is relieved by recombination with the vector to create a packageable genome, containing the gene of choice. The recombinant virus is replication defective in human cells except 293, which express adenovirus E1A and E1B. Following transfection of the two plasmids, infectious virus was recovered, the genomes were analyzed to confirm the recombinant structure, and then virus was plaque purified, all by standard procedures.

As observed with transfection with mda-7, infection of diverse human cancer cell lines, but not normal cell lines, with Ad.mda-7 S inhibited growth. These results demonstrate that this virus retains properties observed with the mda-7 plasmid construct. In many cancer cells, including breast carcinoma (MCF-7 and T47D), glioblastoma (GBM-18 and T98G) and melanoma (HO-1 and C8161), infection with Ad.mda-7 S resulted in the induction of programmed cell death (apoptosis). This effect was not elicited in normal cells even after infection with high multiplicities of infection (100 pfu/cell) with Ad.mda-7 S. In other cancer cell types, growth suppression (as indicated by a suppression in colony formation in monolayer culture) was apparent without signs of apoptosis, as indicated by nuclear morphology changes, formation of nucleosomal ladders or a positive TUNEL reaction. These results indicate that the Ad.mda-7 S virus can selectively inhibit the growth of human cancer cells in vitro. Moreover, in specific cancer cell types growth suppression correlates with induction of apoptosis. These observations suggest that inhibition in cancer growth induced by mda-7 can occur by multiple pathways

Nude mouse human tumor xenograft models were used to determine if Ad.mda-7 S can inhibit the growth of human cancer cells in vivo. Athymic nude mice, obtained from Taconic Labs, were injected subcutaneously with one million human cervical carcinoma (HeLa) cells in PBS mixed with matrigel (final volume 0.4 ml; ratio of matrigel to PBS 1:1). Tumors were allowed to grow until they reached an average volume of 100 to 200 mm³ (10 to 21 days post inoculation). Mice were then randomized and divided into two groups: Group 1: replication-defective Ad lacking the mda-7 gene; null virus (null); and Group 2: Ad.mda-7 S. Treatment consisted of intratumoral injections of the null or Ad.mda-7 S (100 μl at 4 sites/injection) three times a week for 4 weeks. Tumors were measured twice to three times weekly with a caliper. Tumor volumes were calculated using the formula: pi/6×larger diameter×(smaller diameter)². After 4 weeks of therapy, animals were followed for an additional week and sacrificed. Final tumor volume divided by initial tumor volume equals tumor volume ratio which is defined as a measure of cancer progression.

Well-established HeLa xenografts, treated with Ad.mda-7 S, were growth inhibited over the course of the study, whereas tumors treated with the null virus continued to grow progressively (FIGS. 5 and 6). The mda-7 inhibitory effect was significant with a p value<0.05. This study was repeated and similar results were obtained. This data suggest that ectopic expression of mda-7 may provide therapeutic benefit for the treatment of human cancer. Experiments are now in progress using established human breast cancer tumors, MCF-7 and T47D, in nude mice.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 1

<210> SEQ ID NO 1
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

Pro Ser Gln Glu Asn Glu Met Phe Ser le Arg As;
1   5   10

What is claimed is:

1. A method for inducing apoptosis in a tumor cell wherein the tumor cell is from breast cancer, glioblastoma or melanoma, which comprises introducing a nucleic acid comprising a melanoma differentiation associated gene (mda-7) into the cell under conditions permitting the expression of the gene so as to thereby induce apoptosis in the cell.

2. The method of claim 1, wherein the nucleic acid is introduced into the cell via naked DNA technology.

3. The method of claim 1, wherein the nucleic acid is introduced into the cell via an adenovirus vector.

4. The method of claim 1, wherein the nucleic acid is introduced into the cell via an adeno-associated virus vector.

5. The method of claim 1, wherein the nucleic acid is introduced into the cell via an Epstein-Barr virus vector.

6. The method of claim 1, wherein the nucleic acid is introduced into the cell via a Herpes virus vector.

7. The method of claim 1, wherein the nucleic acid is introduced into the cell via an attenuated HIV vector.

8. The method of claim 1, wherein the nucleic acid is introduced into the cell via a retroviral vector.

9. The method of claim 1, wherein the nucleic acid is introduced into the cell via a vaccinia virus vector.

10. The method of claim 1, wherein the nucleic acid is introduced into the cell via a liposome.

11. The method of claim 1, wherein the nucleic acid is introduced into the cell via an antibody-coated liposome.

12. The method of claim 1, wherein the nucleic acid is introduced into the cell via a mechanical means.

13. The method of claim 1, wherein the nucleic acid is introduced into the cell via an electrical means.

14. The method of claim 1, wherein the nucleic acid comprises a vector.

15. The method of claim 14, wherein the vector comprises an adenovirus vector.

16. The method of claim 15, wherein the adenovirus vector is a replication-defective adenovirus vector expressing mda-7.

17. The method of claim 14, wherein the vector comprises an adeno-associated virus vector.

18. The method of claim 14, wherein the vector comprises an Epstein-Barr virus vector.

19. The method of claim 14, wherein the vector comprises a Herpes virus vector.

20. The method of claim 14, wherein the vector comprises an attenuated HIV vector.

21. The method of claim 14, wherein the vector comprises a retrovirus vector.

22. The method of claim 14, wherein the vector comprises a vaccinia virus vector.

23. The method of claim 1, wherein the nucleic acid is linked to a cytomegalovirus promoter.

24. The method of claim 1, wherein the nucleic acid is linked to an RSV promoter.

25. A method for inducing apoptosis in a tumor cell wherein the tumor cell is from breast cancer, glioblastoma or melanoma, which comprises introducing a gene product comprising melanoma differentiation associated-7 (mda-7) protein into the cell so as to thereby induce apoptosis in the cell.

*   *   *   *   *

EXHIBIT 13

APPLICATION FOR UNITED STATES

LETTERS PATENT

for

METHODS FOR TREATMENT OF HYPERPROLIFERATIVE

DISEASES USING HUMAN MDA-7

by

Abner Mhashilkar

Bob Schrock

and

Sunil Chada

EXPRESS MAIL MAILING LABEL
NUMBER: EL548524442US
DATE OF DEPOSIT July 13, 2000

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BACKGROUND OF THE INVENTION

This application claims priority to United States provisional patent application 60/144,354, filed on July 15, 1999 and 60/200,768, filed on April 28, 2000, both of which are specifically incorporated by reference in their entirety herein without disclaimer.

A.   FIELD OF THE INVENTION

The present invention relates generally to the field of gene therapy. More particularly, it concerns a method of administering a therapeutic nucleic acid for the treatment of hyperproliferative diseases. In one embodiment, the invention relates to the expression of a nucleic acid encoding a truncated form of the human MDA-7 (mda7^{TF}) protein for the treatment of hyperproliferative diseases, while in other embodiments the invention involves the full-length form of the human MDA-7 polypeptide for the treatment of hyperproliferative diseases.

B.   DESCRIPTION OF RELATED ART

1.   Gene Therapy

Gene therapy is an emerging field in biomedical research with a focus on the treatment of disease by the introduction of therapeutic recombinant nucleic acids into somatic cells of patients. Various clinical trials using gene therapies have been initiated and include the treatment of various cancers, AIDS, cystic fibrosis, adenosine deaminase deficiency, cardiovascular disease, Gaucher's disease, rheumatoid arthritis, and others. Currently, adenovirus is the preferred vehicle for the delivery of gene therapy agents. Advantages in using adenovirus as a gene therapy agent are high transduction efficiency, infection of non-dividing cells, easy manipulation of its genome, and low probability of non-homologous recombination with the host genome. The present invention describes a novel nucleic acid, encoding a truncated form of human MDA-7 (mda7^{TF}), for the treatment of hyperproliferative disease in humans. Furthermore, the present invention also describes a nucleic acid that encodes a soluble form of the MDA-7 protein and uses thereof.

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2. MDA-7

The cDNA encoding the MDA-7 protein has been described by Jiang *et al.*, 1995 (WO 9511986). The protein encoded by the mda-7 cDNA was recognized as a potential regulator of melanoma progression. Jiang *et al.* used a subtractive hybridization technique (Jiang *et al.*, 1995) to identify genes involved in the regulation of growth and differentiation in human melanoma cells. A cDNA library prepared by subtraction hybridization of cDNAs prepared form actively proliferating human HO-1 melanoma cells against cDNAs prepared from interferon-beta (IFN-β) and mezerin-differentiated human HO-1 melanoma cells was used to identify several melanoma differentiation associated (mda) cDNAs, including mda-7. The expression of mda-7 mRNA is inversely correlated with melanoma progression as demonstrated by increased mRNA levels in normal melanocytes as compared to primary and metastatic melanomas as well as decreased mda-7 mRNA expression in early vertical growth phase melanoma cells selected for enhanced tumor formation in nude mice.

The mda-7 cDNA encodes a novel, evolutionarily conserved protein of 206 amino acids with a predicted size of 23.8 kDa. The deduced amino acid sequence contains a hydrophobic stretch from about amino acid 26 to 45, which has characteristics of a signal sequence. The protein sequence shows no significant amino-acid sequence homology to known proteins or protein motifs with the exception of a 42 amino acid stretch that is 54% identical to interleukin 10 (IL-10). Structural analysis performed by Bazan *et al.* has determined that mda-7 (IL-BKW or IL-20) displays the structural characteristics of the cytokine family (WO 9828425). The structural characteristics and limited identity across a small stretch of amino acids implies an extracellular function for MDA-7.

Additional studies have shown that elevated expression of MDA-7 suppressed cancer cell growth *in vitro* and selectively induced apoptosis in human breast cancer cells as well as inhibiting tumorigenicity in nude mice (Jiang *et al.*, 1996; Su *et al.*, 1998). Jiang *et al.* (1996) report findings that MDA-7 is a potent growth suppressing gene in

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cancer cells of diverse origins including breast, central nervous system, cervix, colon, prostate, and connective tissue. A colony inhibition assay was used to demonstrate that elevated expression of mda-7 enhanced growth inhibition in human cervical carcinoma (HeLa), human breast carcinoma (MCF-7 and T47D), colon carcinoma (LS174T and SW480), nasopharyngeal carcinoma (HONE-1), prostate carcinoma (DU-145), melanoma (HO-1 and C8161), glioblastome multiforme (GBM-18 and T98G), and osteosarcoma (Saos-2). MDA-7 overexpression in normal cells (HMECs, HBL-100, and CREF-Trans6) showed limited growth inhibition indicating that mda-7 transgene effects are not manifest in normal cells. In summary, growth inhibition by elevated expression of MDA-7 is more effective *in vitro* in cancer cells than in normal cells.

Su *et al.* (1998) reported investigations into the mechanism by which MDA-7 suppressed cancer cell growth. The studies reported that ectopic expression of MDA-7 in breast cancer cell lines MCF-7 and T47D induced apoptosis as detected by cell cycle analysis and TUNEL assay without an effect on the normal HBL-100 cells. Western blot analysis of cell lysates from cells infected with adenovirus mda-7 ("Ad-mda-7") showed an upregulation of the apoptosis stimulating protein BAX. Ad-mda-7 infection elevated levels of BAX protein only in MCF-7 and T47D cells and not normal HBL-100 or HMEC cells.

The bax gene plays an important role in inducing apoptosis. Increases in bax transcription may be in part responsible for the p53-regulated pathway of apoptosis-induction (Miyashita *et al.*, 1995). Overexpression of BAX and an increase in the Bax/Bcl-2 protein ratio results in dissipation of mitochondrial membrane potential and release of cytochrome c (Rosse, 1998). The BAX protein binds directly to the mitochondrial porin channel (called voltage dependent anion channel, VDAC) and allows cytochrome c to pass through VDAC (Shimizu *et al.*, 1999). Cytochrome c complexes with Apaf-1 and this complex cleaves and activates caspase-9, an initiator caspase. The caspase cascade is activated from this initiator caspase. In some reported pathways of

cell death, the caspase and Bcl-2 protein families play a key role in the regulation and execution of apoptosis.

Based on the established interactions among the known mediators of apoptosis, three classic pathways of apoptotic signaling in mammalian cell have emerged (Dragovich, 1998). The first one is initiated by the withdrawal of growth factors and is regulated by the Bcl-2 family of proteins. This pathway results in cytochrome c release from mitochondria, activation of Apaf-1 and triggering of the caspase cascade. The other well-established apoptosis pathway involves signaling by cell surface death receptors such as TNF or Fas which, through adapter molecules, can recruit and activate caspases. The third and least well-characterized pathway is initiated by DNA damage. This is regulated in part by proteins such as p53 and ATM . In all three of these pathways of cell death, the caspase and Bcl-2 protein families play key roles in regulation and execution of apoptosis.

3.   Cancer

Normal tissue homeostasis is a highly regulated process of cell proliferation and cell death. An imbalance of either cell proliferation or cell death can develop into a cancerous state (Solyanik *et al.*, 1995; Stokke *et al.*, 1997; Mumby and Walter, 1991; Natoli *et al.*, 1998; Magi-Galluzzi *et al.*, 1998). For example, cervical, kidney, lung, pancreatic, colorectal and brain cancer are just a few examples of the many cancers that can result (Erlandsson, 1998; Kolmel, 1998; Mangray and King, 1998; Gertig and Hunter, 1997; Mougin *et al.*, 1998). In fact, the occurrence of cancer is so high, that over 500,000 deaths per year are attributed to cancer in the United States alone.

The maintenance of cell proliferation and cell death is at least partially regulated by proto-oncogenes. A proto-oncogene can encode proteins that induce cellular proliferation (*e.g., sis, erbB, src, ras and myc*), proteins that inhibit cellular proliferation (*e.g., Rb, p16, p19, p21, p53, NF1 and WT1*) or proteins that regulate programmed cell death *(e.g., bcl-2)* (Ochi *et al.*, 1998; Johnson and Hamdy, 1998; Liebermann *et al.*,

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1998). However, genetic rearrangements or mutations to these proto-oncogenes, results in the conversion of a proto-oncogene into a potent cancer-causing oncogene. Often, a single point mutation is enough to transform a proto-oncogene into an oncogene. For example, a point mutation in the p53 tumor suppressor protein results in the complete

5   loss of wild-type p53 function (Vogelstein and Kinzler, 1992; Fulchi *et al.*, 1998) and acquisition of "dominant" tumor promoting function.

Currently, there are few effective options for the treatment of many common cancer types. The course of treatment for a given individual depends on the diagnosis,

10   the stage to which the disease has developed and factors such as age, sex and general health of the patient. The most conventional options of cancer treatment are surgery, radiation therapy and chemotherapy. Surgery plays a central role in the diagnosis and treatment of cancer. Typically, a surgical approach is required for biopsy and to remove cancerous growth. However, if the cancer has metastasized and is widespread, surgery is

15   unlikely to result in a cure and an alternate approach must be taken. Radiation therapy, chemotherapy and immunotherapy are alternatives to surgical treatment of cancer (Mayer, 1998; Ohara, 1998; Ho *et al.*, 1998). Radiation therapy involves a precise aiming of high energy radiation to destroy cancer cells and much like surgery, is mainly effective in the treatment of non-metastasized, localized cancer cells. Side effects of

20   radiation therapy include skin irritation, difficulty swallowing, dry mouth, nausea, diarrhea, hair loss and loss of energy (Curran, 1998; Brizel, 1998).

Chemotherapy, the treatment of cancer with anti-cancer drugs, is another mode of cancer therapy. The effectiveness of a given anti-cancer drug therapy often is limited by

25   the difficulty of achieving drug delivery throughout solid tumors (el-Kareh and Secomb, 1997). Chemotherapeutic strategies are based on tumor tissue growth, wherein the anti-cancer drug is targeted to the rapidly dividing cancer cells. Most chemotherapy approaches include the combination of more than one anti-cancer drug, which has proven to increase the response rate of a wide variety of cancers (U.S. Patent 5,824,348; U.S.

30   Patent 5,633,016 and U.S. Patent 5,798,339). A major side effect of chemotherapy drugs

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is that they also affect normal tissue cells, with the cells most likely to be affected being those that divide rapidly (*e.g.*, bone marrow, gastrointestinal tract, reproductive system and hair follicles). Other toxic side effects of chemotherapy drugs are sores in the mouth, difficulty swallowing, dry mouth, nausea, diarrhea, vomiting, fatigue, bleeding, hair loss

5   and infection.

Immunotherapy, a rapidly evolving area in cancer research, is yet another option for the treatment of certain types of cancers. For example, the immune system identifies tumor cells as being foreign and thus they are targeted for destruction by the immune

10   system. Unfortunately, the response typically is not sufficient to prevent most tumor growths. However, recently there has been a focus in the area of immunotherapy to develop methods that augment or supplement the natural defense mechanism of the immune system. Examples of immunotherapies currently under investigation or in use are immune adjuvants (*e.g.*, *Mycobacterium bovis*, *Plasmodium falciparum*,

15   dinitrochlorobenzene and aromatic compounds) (U.S. Patent 5,801,005; U.S. Patent 5,739,169; Hui and Hashimoto, 1998; Christodoulides *et al.*, 1998), cytokine therapy (*e.g.*, interferons α, β and γ; IL-1, GM-CSF and TNF) (Bukowski *et al.*, 1998; Davidson *et al.*, 1998; Hellstrand *et al.*, 1998) gene therapy (*e.g.*, TNF, IL-1, IL-2, p53) (Qin *et al.*, 1998; Austin-Ward and Villaseca, 1998; U.S. Patent 5,830,880 and U.S. Patent

20   5,846,945) and monoclonal antibodies (*e.g.*, anti-ganglioside GM2, anti-HER-2, anti-p185) (Pietras *et al.*, 1998; Hanibuchi *et al.*, 1998; U.S. Patent 5,824,311).

As mentioned above, tumor suppressors play an important role in cancer biology. One of these, the p53 tumor suppressor proto-oncogene, is essential for the maintenance

25   of the non-tumorogenic phenotype of cells (reviewed by Soddu and Sacchi, 1998). Approximately 50% of all cancers have been found to be associated with mutations of the p53 gene, which result in the loss of p53 tumor suppressor properties (Levine *et al.*, 1991; Vogelstein and Kinzler, 1992; Hartmann *et al.*, 1996a; Hartmann *et al.*, 1996b). Mutations in the p53 gene also result in the stabilization of the p53 protein in cells with

30   concomitant overexpression of p53 protein. In normal cells, p53 protein is generally

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undetectable due to its high turnover rate. The high incidence of cancer related mutations in the p53 gene has prompted many research groups to investigate p53 as a route of cancer treatment via gene replacement. Ad-mda7 has been shown to suppress the growth of cancer cells that are p53 wildtype, p53 null and p53 mutant. Also, the upregulation of

5   the apoptosis-related *bax* gene indicates that MDA-7 is capable of using p53 independent mechanisms to induce the destruction of cancer cells. These characteristics suggest that MDA-7 has broad therapeutic potential as an anti-proliferative agent.

SUMMARY OF THE INVENTION

10

It is, therefore, an objective of the present invention to provide methods for treating a patient with a hyperproliferative disease comprising administering or giving a therapeutic nucleic acid, such as DNA, encoding either a full-length or truncated human MDA-7 protein or polypeptide under the control of a promoter operable in eukaryotic

15   cells. The therapeutic nucleic acid may be comprised in an expression cassette or construct, which is a nucleic acid molecule capable of allowing the expression of at least a portion of the nucleic acid sequence. A hyperproliferative disease includes diseases and conditions that are associated with any sort of abnormal cell growth or abnormal growth regulation. In methods of the present invention, preferably the patient is a human. The

20   sequence of a full-length MDA-7 polypeptide is provided in SEQ ID. NO:2. A truncated version of MDA-7 would comprise a portion or portions of contiguous amino acid regions of the full-length sequence, but would not contain the entire sequence. The truncated version may be truncated by any number of contiguous amino acids at any site in the polypeptide.

25

The methods for treating a patient with a hyperproliferative disease in the present invention comprise the transfer of a nucleic acid encoding either a full-length or truncated form of the human MDA-7 protein or polypeptide. Following the administration of the nucleic acid to a patient with a hyperproliferative disease, the nucleic acid, under control

30   of a promoter active in eukaryotic cells, is expressed by hyperproliferative cells thereby

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stimulating growth arrest or apoptosis of those cells. Alternatively, the nucleic acid encoding all or part of an MDA-7 protein may be expressed in normal, *i.e.*, non-hyperproliferative, cells and secreted to achieve bystander activity in which neighboring hyperproliferative cells are affected by MDA-7. Thus, it is contemplated that cells that

5   are not hyperproliferative (non-hyperproliferative cells), such as normal or noncancerous cells (*i.e.*, cells that do not exhibit characteristics of unregulated cancerous cell growth), may express a population of MDA-7 protein, some of which is processed into a secretable form that is secreted and taken up by non-transduced (or non-transfected) cells that are nearby. Non-transduced or non-transfected cells are cells that have not internalized the

10   exogenous expression cassette, and thereby, such cells do not express the polypeptide encoded by it. Non-transfected cells could include hyperproliferative or non-normal cells, which may uptake a secreted form of the MDA-7 polypeptide or protein such that the hyperproliferative or non-normal cells are induced to undergo apoptosis or growth inhibited. These non-normal cells may be tumor cells. It is envisioned that non-

15   transduced or non-transfected cells affected by transduced or transfected cells would be proximate to, adjacent to (next to), or near each other. Essentially, the non-tranduced or non-transfected cell is close enough to the transduced or transfected cell so that the MDA-7 protein secreted by the transfected cell reaches the non-transfected cell.

20   In certain embodiments, the hyperproliferative disease is further defined as cancer. In still further embodiments, the cancer is melanoma, non-small cell lung, small-cell lung, lung, hepatocarcinoma, retinoblastoma, astrocytoma, glioblastoma, gum, tongue, leukemia, neuroblastoma, head, neck, breast, pancreatic, prostate, renal, bone, testicular, ovarian, mesothelioma, cervical, gastrointestinal, lymphoma, brain, colon,

25   sarcoma or bladder. The cancer may include a tumor comprised of tumor cells. In other embodiments, the hyperproliferative disease is rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, leiomyomas, adenomas, lipomas, hemangiomas, fibromas, vascular occlusion, restenosis, atherosclerosis, pre-neoplastic lesions (such as adenomatous hyperplasia and prostatic intraepithelial neoplasia), carcinoma *in situ*, oral

30   hairy leukoplakia, or psoriasis.

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In some embodiments, the nucleic acid molecule encodes amino acids from about 49 to about 206; about 75 to about 206; about 100 to about 206; about 125 to about 206; about 150 to about 206; about 175 to about 206; or about 182 to about 206 of SEQ ID NO:2. In some embodiments the expression cassette or vector encodes a truncated MDA-7 polypeptide. Thus, it is contemplated that in some embodiments, the nucleic acid sequence encoding a truncated MDA-7 polypeptide comprises fewer contiguous nucleotides than are in SEQ ID NO:1, *i.e.*, the nucleic acid segment is also less than full-length or truncated. For example, the expression vector or cassette may lack coding sequences corresponding to amino acid 1 to about amino acid 49, amino acid 1 to about amino acid 75, amino acid 1 to about amino acid 100, amino acid 1 to about amino acid 125, amino acid 1 to about amino acid 150, amino acid 1 to about amino acid 175, or amino acid 1 to about amino acid 182, of SEQ ID NO:2.

Nucleic acid molecules of the present invention may contain sequences encoding a full-length, human mda-7 gene, as disclosed in SEQ ID NO:1. In some embodiments of the invention, a nucleic acid molecule may encode fewer nucleotides than is depicted in SEQ ID NO:1, such that the molecule contains fewer than 700 contigous nucleotides from SEQ ID NO:1. In some aspects, a nucleic acid molecule may contain about 50, 100, 200, 300, 400, 500, 600, or 700 contigous nucleotides from SEQ ID NO:1. Alternatively the molecule may encode a nucleic acid molecule that encodes a MDA-7 polypeptide missing the first 49 amino acids of SEQ ID NO:2 because the nucleic acid sequence corresponding to the first 49 amino acids is absent.

In certain other embodiments, the nucleic acid further comprises nucleotides encoding a heterologous secretory signal sequence in which the heterologous sequence is derived from a non-MDA-7 nucleic acid sequence or polypeptide. A "signal sequence" refers to a sequence, typically short, that directs a newly translated secretory or membrane polypeptide to and through the endoplasmic reticulum or across a membrane. The signal sequence allows a protein to be secreted from a cell. In further embodiments, the nucleic

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acid further comprises a heterologous secretory signal sequence defined as a positively charged N-terminal region in combination with a hydrophobic core.

In certain embodiments, the promoter is CMV IE, dectin-1, dectin-2, human CD11c, F4/80, SM22, RSV, SV40, Ad MLP, beta-actin, MHC class I or MHC class II promoter, however any other promoter that is useful to drive expression of the mda-7 gene of the present invention, such as those set forth hereinbelow, is believed to be applicable to the practice of the present invention. In other embodiments, a polyadenylation signal is operatively linked to a MDA-7 coding region.

In certain embodiments, the nucleic acid is a viral vector, wherein the viral vector dose is from about 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13} pfu and higher. Alternatively, dosage may be expressed in units of viral particles (vp); thus, the numbers listed above in "pfu" units may be expressed in units of "vp" units or "viral particles." It is contemplated that about 10^3 to about 10^{15}, about 10^5 to about 10^{12}, or 10^7 to about 10^{10} viral particles may be administered to a patient.

In some embodiments, the viral vector is an adenoviral vector, a retroviral vector, a vaccinia viral vector, an adeno-associated viral vector, a polyoma viral vector, alpha viral vector or a herpesviral vector. In some aspects, the viral vector is an adenoviral vector. It is contemplated that the viral vector may be replication-deficient or -defective. While in other embodiments, an adenovirus vector that contains Ad-5 sequences may be employed; in some aspects, an adenovirus vector construct lacks E1-coding regions, which may comprise a deletion of both E1A and E1B sequences.

The methods of the present invention include dispersing expression constructs, vectors, and cassettes in pharmacologically acceptable solution for administration to a patient. In some cases, the pharmacologically acceptable solution comprises a lipid. In further embodiments of the present invention, a nucleic acid molecule encoding a full-length or truncated MDA-7 polypeptide is administered in a lipoplex (*i.e.*, as a lipid-

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nucleic acid complex), which may contain, as described in some embodiments, DOTAP and at least one cholesterol, cholesterol derivative, or cholesterol mixture. These nucleic acid molecules may be administered to the patient intravenously, intraperitoneally, intratracheally, intratumorally, intramuscularly, endoscopically, intralesionally, percutaneously, subcutaneously, regionally, or by direct injection or perfusion. It is further contemplated that treatment methods may involve multiple administrations.

The nucleic acid of the present invention may be administered by injection. Other embodiments include the administering of the nucleic acid by multiple injections. In certain embodiments, the injection is performed local, regional or distal to a disease or tumor site. In some embodiments, the administering of nucleic acid is via continuous infusion, intratumoral injection, or intravenous injection. In certain other embodiments, the nucleic acid is administered to the tumor bed prior to or after; or both prior to and after resection of the tumor.

In some embodiments of the present invention includes combination therapy methods using a nucleic acid sequence encoding a full-length or truncated MDA-7 polypeptide in combination with a second therapy to treat a hyperproliferative disease. In cases involving cancer, the nucleic acid molecule may be administered to the patient before, during, or after chemotherapy, biotherapy such as gene therapy with a second therapeutic polynucleotide other than a polynucleotide encoding an MDA-7 polypeptide), immunotherapy, surgery or radiotherapy.

While in further embodiments, chemotherapy involving at least one DNA damaging agent is implemented in combination with administration of an MDA-7 encoding nucleic acid molecule. The DNA damaging agent may be gamma-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), or hydrogen peroxide. In further embodiments, the DNA damaging agent is adriamycin. While in other embodiments, the chemotherapy comprises a cisplatin

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(CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, taxotere, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin, or methotrexate or any analog

5   or derivative variant thereof. In one aspect of the invention, the chemotherapy comprises tamoxifen, while in another aspect is it comprises adriamycin. Further embodiments involve immunotherapy, such as Herceptin.

In cases involving a cancerous tumor, a combination treatment may involve

10   administration of a nucleic acid molecule encoding a full-length or truncated MDA-7 polypeptide and tumor resection, which may occur before, after, or during the mda-7 gene therapy administration. If mda-7 treatment occurs after tumor resection, the expression construct or vector encoding MDA-7 may be administered to the tumor bed.

15   Other methods of the invention include treating a patient with a hyperproliferative disease in a process involving at least the following step: administering to the patient an adenovirus composition that contains an adenovirus construct with a human mda-7 gene under the control of a promoter in an amount effective to confer a therapeutic benefit on the patient. Another aspect includes methods of inducing apoptosis in a cancer cell by

20   administering to a cancer cell in a subject an expression cassette containing a nucleic acid sequence encoding a human MDA-7 protein under the control of a promoter operable in eukaryotic cells. In yet further embodiments, the invention includes methods of inducing apoptosis in a cancer cell by administering to a noncancerous cell in a subject an expression cassette that contains a nucleic acid sequence encoding a human MDA-7

25   polypeptide under the control of a promoter operable in eukaryotic cells, wherein the MDA-7 polypeptide is expressed and secreted. With these types of bystander methods, a transfected noncancerous cell may be adjacent or close enough to a cancer cell such that the noncancerous cell secretes an MDA-7 polypeptide that induces growth arrest or apoptosis in the untransfected cancer cell. Other methods include those directed at

30   treating a patient with cancer by administering to a noncancerous cell in the patient an

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effective amount of an adenovirus composition to confer a therapeutic benefit on that patient. This adenovirus composition can include an adenovirus vector construct that contains a mda-7 gene under the control of a promoter.

5   The methods of the present invention also encompass methods of treating a tumor by inducing apoptosis in transfected and untransfected tumor cells comprising administering to the tumor an adenovirus composition comprising an adenovirus vector construct comprising a human mda-7 gene under the control of a promoter, such that transfected cells express and secrete a truncated MDA-7 polypeptide. And still other

10   embodiments include methods of treating cancer by administering to a subject with cancer an adenovirus composition that contains an adenovirus vector construct with a human mda-7 gene under the control of a promoter to a cell that does not have mutated p53, Rb, ras, or p16 genes, in an amount effective to induce apoptosis in a cell that does have a mutated p53, Rb, ras, or p16 gene.

15

Other methods of the invention include treating a subject with a tumor by administering to the subject a nucleic acid molecule comprising a human mda-7 gene under the control of a promoter in an amount effective to inhibit angiogenesis around the tumor. Such methods may also include steps to evaluate the level of angiogenesis

20   inhibition. It is contemplated that other embodiments of treatment described herein may be implemented with these methods.

Compositions of the present invention include an expression vector encoding a mda-7 coding region under the control of a promoter operable in an eukaryotic cell, such

25   that the coding region contains a deletion corresponding to N-terminal sequences. The expression vector compositions may include any expression cassette described with respect to the methods of the present invention. Similarly, any compositions described herein may be utilized in the practice of any of the methods disclosed herein.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

5   Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those

10   skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

The following drawings form part of the present specification and are included to

15   further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Schematic illustration of Ad-vectors. Replication-deficient human type 5

20   Adenovirus (Ad5) carrying the mda-7 (or luciferase gene) linked to an internal CMVIE promoter and followed by SV40 polyadenylation (pA) signal were used. In addition, Ad-CMVp(A) (empty vector) was used as control.

FIG 2A. T47D cells treated with Ad-mda7 at varying MOIs (viral particle/cell). **FIG**

25   **2B.** MCF-7 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell).

FIG. 3A. MDA-MB-361 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell). **FIG. 3B.** BT-20 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell).

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FIG. 4A. H1299 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell). **FIG. 4B.** H322 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell).

5 **FIG. 5A.** SW620 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell). **FIG. 5B.** DLD-1 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell).

FIG. 6A. MJ90 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell). **FIG. 6B.** HUVEC cells treated with Ad-mda7 at varying MOIs (Viral particle/cell).

10

FIG. 7. Annexin V assay to determine apoptosis induction after Ad-mda7 transduction in breast cancer cell lines. Three breast cancer cell lines (T47D, MDA-MB-468, MCF-7) were infected with Ad-mda7 or control Ad-CMVp(A) empty vector, and evaluated for apoptosis using Annexin V.

15

FIG. 8. DLD-1 cells were infected with Ad-mda7 or Ad-luc and 48 hours later examined for Annexin V staining by FACS analysis.

FIG. 9. Panel A shows apoptosis induction in H1299 cells infected with Ad-mda7 or Ad-
20 luc. Cells were evaluated at different time points post-infection using Annexin V staining and FACS analysis. Panel B illustrates apoptosis in DLD-1 cells that were infected with Ad-mda7 or Ad-luc at different time points post-infection (as examined by Annexin V staining and FACS analysis).

25 **FIG. 10.** H460 cells were infected with increasing MOIs of Ad-mda7 or Ad-luc and 48 h later processed for MDA7 surface expression and analyzed by FACS.

FIG. 11A. Soluble MDA-7 (sMDA7) kills tumor cells. H1299 cells were challenged with the following samples and percentage dead cells evaluated after 48 hours: 1) Ad-
30 mda7 virus, positive control infected at 1000 Vp/cell; 2) Soluble MDA7 supernatant from

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H1299 infected cells with Ad-mda-7 (1000 vp/cell); 3) Ad-luc virus, control infected at 1000 Vp/ cell; 4) supernatant from H1299 infected cells with Ad-luc (1000 vp/cell); 5) Ad-p53 virus, positive control infected at 20 Vp/cell; 6) a separate stock of soluble MDA-7 supernatant obtained from 293 cells infected with Ad-mda-7 (sup M, 500 Vp/cell); and

5   7) a separate stock of soluble MDA-7 supernatant obtained from modified serum-free 293 cells infected with Ad-mda-7 (sup P, 500 Vp/cell). All the supernatants used in this experiment were filtered through a 0.1 micron filter prior to challenge with H1299 cells. **FIG. 11B.** H1299 cells were challenged with soluble MDA-7 supernatant from four different stocks and percentage dead cells evaluated after 48 hours: 1) 293*NF: Non-

10   filtered supernatant obtained from modified 293 cells (cells were grown in serum-free conditions); 2) 293*F: 0.1 micron filtered supernatant obtained from modified 293 cells; 3) 293F: 0.1 micron filtered supernatant obtained from regular 293 cells (FBS +); and 4) H1299F: 0.1 micron filtered supernatant obtained from H1299 cells. D0 is non-diluted material whereas D1:1; D1:5, D1:10 indicate the dilutions used. Control undiluted

15   supernatant from Ad-luc treated H1299 cells demonstrated 20% dead cells.

FIG. 12. Combination with Tamoxifen. Ad-mda7 has been combined with tamoxifen and evaluated for anti-tumor effects in breast cancer cell lines. The graphs demonstrate that combining these two agents provides superior anti-tumor activity compared to either

20   agent alone.

FIG. 13. Combination with Adriamycin. Ad-mda7 has been combined with adriamycin and evaluated for anti-tumor effects in breast cancer cell lines. The graphs demonstrate that combining these two agents provides superior anti-tumor activity compared to either

25   agent alone.

FIG. 14. Left Panel: MDA-7 protein expression in NSCLC cells and normal lung cells after transduction with Ad-mda7. NHFB-normal human bronchial cells. **Right Panel-upper:** Effect of Ad-mda7 on growth of NSCLC cells and normal lung cells. Ad-mda7

30   (circles), PBS (diamonds), Ad-luc (squares). **Lower Panel:** Cell cycle analysis of

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NSCLC cells and normal lung cells after transduction with Ad-mda7. Note significant decrease in G1 and increase in G2/M.

FIG. 15. Combination of Ad-mda7 and Herceptin on breast cancer cell lines. Cell lines treated with Ad-mda7 are enhanced in a Her2-expressing cell line as compared to a non-expressing cell line, demonstrating the increased effectiveness of Herceptin on killing cells following contact with Ad-mda7.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention contemplates the treatment of hyperproliferative diseases by identifying patients with such diseases and expressing either a full-length or truncated form of human MDA-7 polypeptide in these hyperproliferative cells or normal cells neighboring hyperproliferative cells by means of nucleic acid transfer. The treatment of such a hyperproliferative disease in one embodiment involves the intratumoral administration of a full-length or truncated human MDA-7 expression construct to hyperproliferative cells. The hyperproliferative cells or normal cells then express a full-length or truncated form of human MDA-7, resulting in the growth inhibition or death of the hyperproliferative cells. Furthermore, neighboring hyperproliferative cells that have not taken up the MDA-7 expression construct may also be growth inhibited and/ or killed by the soluble form of MDA-7.

A.   HYPERPROLIFERATIVE DISEASE AND MDA-7

A variety of hyperproliferative diseases can be treated according to the methods of the present invention. Some of the hyperproliferative diseases contemplated for treatment in the present invention are psoriasis, rheumatoid arthritis (RA), inflammatory bowel disease (IBD), osteoarthritis (OA) and pre-neoplastic lesions in the mouth, prostate, breast, lung etc.. The present invention has important ramifications particularly with respect to one hyperproliferative disease: cancer.

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Cancer has become one of the leading causes of death in the western world, second only behind heart disease. Current estimates project that one person in three in the U.S. will develop cancer, and that one person in five will die from cancer. Cancers can be viewed as altered cells that have lost the normal growth-regulating mechanisms.

5

There currently are three major categories of oncogenes, reflecting their different activities. One category of oncogenes encodes proteins that induce cellular proliferation. A second category of oncogenes, called tumor-suppressors genes or anti-oncogenes, function to inhibit excessive cellular proliferation. The third category of oncogenes,

10   either block or induce apoptosis by encoding proteins that regulate programmed cell death.

The cDNA encoding the MDA-7 protein has been described by Jiang *et al.*, 1995 (WO 9511986). The protein encoded by the mda-7 cDNA was recognized as a potential

15   regulator of melanoma progression. Jiang *et al.* used a subtractive hybridization technique (Jiang *et al.*, 1995) to identify genes involved in the regulation of growth and differentiation in human melanoma cells. A subtracted cDNA library prepared by subtraction hybridization of cDNAs prepared form actively proliferating human HO-1 melanoma cells against cDNAs prepared from interferon (IFN-β) and mezerin

20   differentiated human HO-1 melanoma cells was used to identify several melanoma differentiation associated (mda) cDNAs. The cDNA for MDA-7 was identified as having elevated expression levels in the differentiated melanoma cells.

That MDA-7 increased BAX levels in cancer cell lines led to an evaluation of the

25   effect of *ex vivo* Ad-mda-7 transduction on xenograft tumorigenicity of MCF-7 tumor cells. *Ex vivo* transduction resulted in the inhibition of tumor formation and progression in the tumor xenograft model.

Treatment of cells with a mda-7 expression vector results in the secretion of a

30   soluble form of the MDA-7 protein. This soluble protein possesses anti-tumor activity.

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Therefore, the combination of direct induction of apoptosis and release of soluble mediator with anti-tumor properties will provide enhanced activity against hyperproliferative diseases. The cancer cell-specific anti-proliferative effects of elevated MDA-7 expression make this molecule an ideal gene therapy treatment for hyperproliferative disease, especially cancer.

In some embodiments, the treatment of a wide variety of cancerous states or tissue/organ types is within the scope of the invention, for example, melanoma, non-small cell lung, small-cell lung, lung, hepatocarcinoma, retinoblastoma, astrocytoma, glioblastoma, leukemia, blood, brain, skin, eye, tongue, gum, neuroblastoma, head, neck, breast, pancreatic, prostate, renal, bone, testicular, ovarian, mesothelioma, cervical, gastrointestinal, lymphoma, brain, colon or bladder. In still more preferred embodiments the hyperproliferative disease being treated according to the present invention is rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, leiomyomas, adenomas, lipomas, hemangiomas, fibromas, vascular occlusion, restenosis, atherosclerosis, pre-neoplastic lesions, carcinoma *in situ*, oral hairy leukoplakia or psoriasis.

The present invention is directed at the use of at least a part of an MDA-7 protein to treat patients with hyperproliferative diseases such that these patients are conferred a therapeutic benefit as a result of the treatment. The term "therapeutic benefit" used throughout this application refers to anything that promotes or enhances the well-being of the patient with respect to the medical treatment of his hyperproliferative disease. A list of nonexhaustive examples of this includes extension of the patient's life by any period of time; decrease or delay in the neoplastic development of the disease; decrease in hyperproliferation; reduction in tumor growth; delay of metastases; reduction in the proliferation rate of a cancer cell, tumor cell, or any other hyperproliferative cell; induction of apoptosis in any treated cell or in any cell affected by a treated cell; and a decrease in pain to the patient that can be attributed to the patient's condition.

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1. MDA Protein, Polypeptides, and Peptides

The mda-7 cDNA encodes a novel, evolutionarily conserved protein of 206 amino acids with a predicted size of 23.8 kDa. The deduced amino acid sequence contains a hydrophobic stretch from about amino acid 26 to 45. The protein sequence shows no

5   significant homology to known proteins or protein motifs with the exception of a 42 amino acid stretch that is 54% identical to interleukin 10 (IL-10). Structural analysis performed by Bazan *et al.* has determined that a shortened soluble form of MDA-7 (IL-BKW or IL-20) displays structural characteristics of the cytokine family (WO 9828425) and antagonizes IL-10 function. Bazan et al. note that the coding region of the mda-7

10   cDNA was mis-identified (p.6, 1.30). Furthermore, they assert that the "pre-sequence of IL-BKW/mda-7 probably starts at either the M(et) at position 28 or 30..(p.9, 1.3)" of the MDA-7 sequence. The structural characteristics and limited identity across a small stretch of amino acids implies a potential extracellular function for MDA-7. The inventors demonstrated that Ad-mda7, which encodes the full length 206 amino-acid

15   sequence, gives rise to an intracellular protein of approximately 23 kD. Furthermore, the Ad-mda7 vector also causes release of a soluble form of MDA-7 protein from treated cells. The soluble MDA-7 protein is approximately 40kD and is glycosylated. Treatment with glycosidases reduces the molecular mass of the soluble protein. Inhibitors of protein secretion, brefeldin A and tunicamycin, cause an intracellular accumulation of MDA-7

20   and inhibit release of this protein from cells. The MDA-7 soluble protein causes growth inhibition of tumor cells. Therefore, release of this soluble MDA-7 can give rise to a "bystander" effect wherein tumor cells that are not contacted by a mda-7 expression construct will be growth inhibited.

25   Additional studies have shown that elevated expression of MDA-7 suppressed cancer cell growth and selectively induced apoptosis in human breast cancer cells as well as inhibiting tumorigenicity in nude mice (Jiang *et al.*, 1996 and Su *et al.*, 1998). Jiang *et al.* (1996) report findings that mda-7 is a potent growth suppressing gene in cancer cells of diverse origins including breast, central nervous system, cervix, colon, prostate, and

30   connective tissue. A colony inhibition assay was used to demonstrate that elevated

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expression of mda-7 enhanced growth inhibition in human cervical carcinoma (HeLa), human breast carcinoma (MCF-7 and T47D), colon carcinoma (LS174T and SW480), nasopharyngeal carcinoma (HONE-1), prostate carcinoma (DU-145), melanoma (HO-1 and C8161), glioblastome multiforme (GBM-18 and T98G), and osteosarcoma (Saos-2). Mda-7 overexpressed in normal cells (HMECs, HBL-100, and CREF-Trans6) did not show significant effects.

Growth inhibition by elevated expression of MDA-7 is more effective in cancer cells than in normal cells. Su *et al.* (1998) investigated the mechanism by which MDA-7 suppressed cancer cell growth. The studies reported that ectopic expression of MDA-7 in breast cancer cell lines MCF-7 and T47D induced apoptosis as detected by cell cycle analysis and TUNEL assay without an effect on the normal HBL-100 cells. Western blotting of lysates from cells infected with adenovirus mda-7 showed an upregulation of the apoptosis stimulating protein BAX. Ad-mda-7 infection elevated levels of BAX protein only in MCF-7 and T47D cells and not normal HBL-100 or HMEC cells. These data led the investigators to evaluate the effect of *ex vivo* Ad-mda-7 transduction on xenograft tumorigenicity of MCF-7 tumor cells. *Ex vivo* transduction resulted in the inhibition of tumor formation and progression in the tumor xenograft model. MDA-7 has been shown to be effective in tumor cell-specific apoptotic induction. Thus, one embodiment of the present invention is the treatment of various hyperproliferative diseases with a mda-7 adenoviral construct encoding full-length or truncated MDA-7.

Of particular interest, according to the present invention, is the use of a soluble form of MDA-7. WO 98/28425 describes a cytokine molecule allegedly related to IL-10. This molecule, designated IL-BKW, appears to be derived from the same gene as MDA-7. However, the authors describe the coding region designation of MDA-7 as "mis-identified". The mature form of IL-BKW was to begin at about residue 47 or 49 of the mda-7 coding region, and continue some 158-160 residues, *i.e.*, to residues 206 of the mda-7 sequence. Thus, a preferred molecule would preferably lack all or part of both the

putative signal sequence (residues 1-25) and a putative membrane spanning hydrophobic domain (residues 26-45) of full length MDA-7.

Other even shorter molecules are contemplated. For example, while molecules
5 beginning approximately at MDA-7 residues 46-49 are the largest molecules, further N-terminal truncations are within the scope of the invention. Thus, specifically contemplated are molecules starting at residue 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102,
10 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, and 182, and terminating at residue 206.
15

Though not adhering to a particular theory regarding the operability of these constructs, there is a notable homology between MDA-7 and IL-10, as well as across species in the C, E, and F helical regions and also in the amino acid positions that have been implicated in receptor binding. The tertiary structure of MDA-7 closely models the
20 known structure of IL-10, further suggesting that these molecules are related. Thus, molecules containing any or all of these amino acid regions are particularly preferred.

Alternatively, in other embodiments a full-length or a substantially full-length MDA-7 polypeptide is contemplated to be of use in the treatment of hyperproliferative
25 diseases and conditions. When used in the context of human MDA-7, the term "full-length" refers to a MDA-7 polypeptide that contains at least the 206 amino acids encoded by the human mda-7 cDNA. The term "substantially full-length" in the context of human MDA-7 refers to a MDA-7 polypeptide that contains at least 80% of the contiguous amino acids of the full-length human MDA-7 polypeptide (SEQ ID NO:2). However, it
30 is also contemplated that MDA-7 polypeptides containing at least about 85%, 90%, and

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95% of SEQ ID NO:2 are within the scope of the invention as "substantially full-length" MDA-7. A "truncated MDA-7 polypeptide" or "truncated MDA-7" refers to an MDA-7 polypeptide that is lacking contiguous amino acids from the full-length MDA-7 amino acid sequence. The missing contiguous amino acids could number *inter alia* about 3, 4,

5   5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more amino acids. The phrase "secreted MDA-7" refers to an MDA-7 polypeptide that is secreted from a cell, *i.e.*, a polypeptide that may or may not be encoded by a full-length mda-7 cDNA and whose N-terminus

10   begins at about amino acid 46 of the full-length MDA-7 polypeptide. The phrases "truncated MDA-7" and "truncated MDA-7 polypeptide" include a secreted MDA-7 polypeptide if, for example, the signal sequence is not missing or if a heterologous signal sequence is attached to the truncated polypeptide.

15   The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein. Accordingly, a sequence that has between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:2 will be a sequence that is

20   "essentially as set forth in SEQ ID NO:2," provided the biological activity of the protein, polypeptide, or peptide is maintained.

The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine and serine, and also

25   refers to codons that encode biologically equivalent amino acids.

Excepting intronic and flanking regions, and allowing for the degeneracy of the genetic code, nucleic acid sequences that have between about 70% and about 79%; or more preferably, between about 80% and about 89%; or even more particularly, between

30   about 90% and about 99%; of nucleotides that are identical to the nucleotides of

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SEQ ID NO:1 will be nucleic acid sequences that are "essentially as set forth in SEQ ID NO:1."

It will also be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NO:1 and SEQ ID NO:2, respectively. Recombinant vectors and isolated nucleic acid segments may therefore variously include these coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, and they may encode larger polypeptides or peptides that nevertheless include such coding regions or may encode biologically functional equivalent proteins, polypeptide or peptides that have variant amino acids sequences.

The nucleic acids of the present invention encompass biologically functional equivalent MDA-7 proteins, polypeptides, or peptides. Such sequences may arise as a consequence of codon redundancy or functional equivalency that are known to occur naturally within nucleic acid sequences or the proteins, polypeptides or peptides thus encoded. Alternatively, functionally equivalent proteins, polypeptides or peptides may be created via the application of recombinant DNA technology, in which changes in the protein, polypeptide or peptide structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Recombinant changes may be introduced, for example, through the application of site-directed mutagenesis techniques as discussed herein below, e.g., to introduce improvements or alterations to the antigenicity of the protein, polypeptide or peptide, or to test mutants in order to examine MDA-7 protein, polypeptide, or peptide activity at the molecular level.

Fusion proteins, polypeptides or peptides may be prepared, e.g., where the mda-7 coding regions are aligned within the same expression unit with other proteins, polypeptides or peptides having desired functions. Non-limiting examples of such desired functions of expression sequences include purification or immunodetection purposes for the added expression sequences, e.g., proteinaceous compositions that may

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be purified by affinity chromatography or the enzyme labeling of coding regions, respectively.

Encompassed by the invention are nucleic acid sequences encoding relatively small peptides or fusion peptides, such as, for example, peptides of from about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, about 90, about 91, about 92, about 93, about 94, about 95, about 96, about 97, about 98, about 99, to about 100 amino acids in length, or more preferably, of from about 15 to about 30 amino acids in length; as set forth in SEQ ID NO:2 and also larger polypeptides up to and including proteins corresponding to the full-length sequences set forth in SEQ ID NO:2.

2.   mda-7 Nucleic Acids and Uses Thereof

One embodiment of the present invention is to transfer the nucleic acids encoding the full-length, substantially full-length, or truncated form of human MDA-7 to induce the destruction, apoptosis or lysis of hyperproliferative cells. The expression of MDA-7 is inversely correlated with tumor progression as demonstrated by increased mRNA levels in normal melanocytes as compared to primary and metastatic tumors as well as decreased MDA-7 expression in early vertical growth phase tumor cells selected for enhanced tumor formation in nude mice.

Thus, in some embodiments of the present invention, the treatment of hyperproliferative disease involves the administration of a therapeutic nucleic acid expression construct encoding a full-length, substantially full-length, or truncated form of MDA-7 to hyperproliferative cells. It is contemplated that the hyperproliferative cells take up the construct and express the therapeutic polypeptide encoded by nucleic acid, thereby restoring a growth control to or destroying the hyperproliferative cells. Furthermore, the soluble MDA-7 released from transfected or transduced cells will be available locally and provide a bystander effect on neighboring tumor cells. Thus, it is contemplated that the therapeutic mda-7 expression construct may be delivered to normal cells and the released bystander effector (MDA-7, full-length or truncated) would cause anti-tumor effects, particularly with respect to hyperproliferative cells.

Certain aspects of the present invention concern at least one human mda-7 nucleic acid molecule. In certain aspects, the mda-7 nucleic acid comprises a wild-type or mutant mda-7 nucleic acid. In particular aspects, the mda-7 nucleic acid encodes for at least one transcribed nucleic acid. In particular aspects, the mda-7 nucleic acid encodes at least one MDA-7 protein, polypeptide, or peptide, or biologically functional equivalent thereof. In other aspects, the human mda-7 nucleic acid comprises at least one nucleic acid segment of SEQ ID NO:1 or at least one biologically functional equivalent thereof.

The present invention also concerns the isolation or creation of at least one recombinant construct or at least one recombinant host cell through the application of recombinant nucleic acid technology known to those of skill in the art or as described herein. The recombinant construct or host cell may comprise at least one mda-7 nucleic acid, and may express at least one MDA-7 protein, polypeptide, or peptide, or at least one biologically functional equivalent thereof.

As used herein "wild-type" refers to the naturally occurring sequence of a nucleic acid at a genetic locus in the genome of an organism, and sequences transcribed or translated from such a nucleic acid. Thus, the term "wild-type" also may refer to the

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amino acid sequence encoded by the nucleic acid. As a genetic locus may have more than one sequence or alleles in a population of individuals, the term "wild-type" encompasses all such naturally occurring alleles. As used herein the term "polymorphic" means that variation exists (*i.e.*, two or more alleles exist) at a genetic locus in the

5   individuals of a population. As used herein, "mutant" refers to a change in the sequence of a nucleic acid or its encoded protein, polypeptide, or peptide that is the result of recombinant DNA technology.

A nucleic acid may be made by any technique known to one of ordinary skill in

10   the art. Non-limiting examples of synthetic nucleic acid, particularly a synthetic oligonucleotide, include a nucleic acid made by *in vitro* chemical synthesis using phosphotriester, phosphite or phosphoramidite chemistry and solid phase techniques such as described in EP 266,032, incorporated herein by reference, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.*, 1986, and U.S. Patent Serial

15   No. 5,705,629, each incorporated herein by reference. A non-limiting example of enzymatically produced nucleic acid include one produced by enzymes in amplification reactions such as PCR™ (see for example, U.S. Patent 4,683,202 and U.S. Patent 4,682,195, each incorporated herein by reference), or the synthesis of oligonucleotides described in U.S. Patent No. 5,645,897, incorporated herein by reference. A non-limiting

20   example of a biologically produced nucleic acid includes recombinant nucleic acid production in living cells, such as recombinant DNA vector production in bacteria (see for example, Sambrook *et al.* 1989, incorporated herein by reference).

A nucleic acid may be purified on polyacrylamide gels, cesium chloride

25   centrifugation gradients, or by any other means known to one of ordinary skill in the art (see for example, Sambrook *et al.* 1989, incorporated herein by reference).

The term "nucleic acid" will generally refer to at least one molecule or strand of DNA, RNA or a derivative or mimic thereof, comprising at least one nucleobase, such as,

30   for example, a naturally occurring purine or pyrimidine base found in DNA (*e.g.*, adenine

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"A," guanine "G," thymine "T," and cytosine "C") or RNA (*e.g.* A, G, uracil "U," and C). The term "nucleic acid" encompasses the terms "oligonucleotide" and "polynucleotide." The term "oligonucleotide" refers to at least one molecule of between about 3 and about 100 nucleobases in length. The term "polynucleotide" refers to at least one molecule of

5   greater than about 100 nucleobases in length. These definitions generally refer to at least one single-stranded molecule, but in specific embodiments will also encompass at least one additional strand that is partially, substantially or fully complementary to the at least one single-stranded molecule. Thus, a nucleic acid may encompass at least one double-stranded molecule or at least one triple-stranded molecule that comprises one or more

10   complementary strand(s) or "complement(s)" of a particular sequence comprising a strand of the molecule.

In certain embodiments, a "gene" refers to a nucleic acid that is transcribed. As used herein, a "gene segment" is a nucleic acid segment of a gene. In certain aspects, the

15   gene includes regulatory sequences involved in transcription, or message production or composition. In particular embodiments, the gene comprises transcribed sequences that encode for a protein, polypeptide or peptide. In other particular aspects, the gene comprises a mda-7 nucleic acid, and/or encodes a MDA-7 polypeptide or peptide-coding sequences. In keeping with the terminology described herein, an "isolated gene" may

20   comprise transcribed nucleic acid(s), regulatory sequences, coding sequences, or the like, isolated substantially away from other such sequences, such as other naturally occurring genes, regulatory sequences, polypeptide or peptide encoding sequences, etc. In this respect, the term "gene" is used for simplicity to refer to a nucleic acid comprising a nucleotide sequence that is transcribed, and the complement thereof. In particular

25   aspects, the transcribed nucleotide sequence comprises at least one functional protein, polypeptide and/or peptide encoding unit. As will be understood by those in the art, this functional term "gene" includes both genomic sequences, RNA or cDNA sequences, or smaller engineered nucleic acid segments, including nucleic acid segments of a non-transcribed part of a gene, including but not limited to the non-transcribed promoter or

30   enhancer regions of a gene. Smaller engineered gene nucleic acid segments may express,

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or may be adapted to express using nucleic acid manipulation technology, proteins, polypeptides, domains, peptides, fusion proteins, mutants and/or such like. Thus, a "truncated gene" refers to a nucleic acid sequence that is missing a stretch of contiguous nucleic acid residues that encode a portion of the full-length MDA-7 polypeptide. For example, a truncated gene may not contain the nucleic acid sequence for the N-terminal region of the MDA-7 polypeptide, such as the first 46 amino acids. It is envisioned that the nucleic acid sequences of the present invention may contain fewer than 95% of the contiguous nucleic acid residues of SEQ ID NO:1. Alternatively, these sequences may encode fewer than 90%, 85%, 80%, 75%, or 70% of the contiguous nucleic acid residues of SEQ ID NO:1.

"Isolated substantially away from other coding sequences" means that the gene of interest, in this case the mda-7 gene, forms the significant part of the coding region of the nucleic acid, or that the nucleic acid does not contain large portions of naturally-occurring coding nucleic acids, such as large chromosomal fragments, other functional genes, RNA or cDNA coding regions. Of course, this refers to the nucleic acid as originally isolated, and does not exclude genes or coding regions later added to the nucleic acid by recombinant nucleic acid technology.

In certain embodiments, the nucleic acid is a nucleic acid segment. As used herein, the term "nucleic acid segment," are smaller fragments of a nucleic acid, such as for non-limiting example, those that encode only part of the MDA-7 peptide or polypeptide sequence. Thus, a "nucleic acid segment may comprise any part of the mda-7 gene sequence, of from about 2 nucleotides to the full-length of the MDA-7 peptide- or polypeptide-encoding region. In certain embodiments, the "nucleic acid segment" encompasses the full-length mda-7 gene sequence. In particular embodiments, the nucleic acid comprises any part of SEQ ID NO:1 of from about 2 nucleotides to the full-length of the sequence encoding SEQ ID NO:2.

Various nucleic acid segments may be designed based on a particular nucleic acid sequence, and may be of any length. By assigning numeric values to a sequence, for example, the first residue is 1, the second residue is 2, *etc.*, an algorithm defining all nucleic acid segments can be created:

5

$$n \text{ to } n + y$$

where n is an integer from 1 to the last number of the sequence and y is the length of the nucleic acid segment minus one, where n + y does not exceed the last number of the sequence. Thus, for a 10-mer, the nucleic acid segments correspond to bases 1 to 10, 2 to 11, 3 to 12 ... and/or so on. For a 15-mer, the nucleic acid segments correspond to bases 1

10   to 15, 2 to 16, 3 to 17 ... and/or so on. For a 20-mer, the nucleic segments correspond to bases 1 to 20, 2 to 21, 3 to 22 ... and/or so on. In certain embodiments, the nucleic acid segment may be a probe or primer.

The nucleic acid(s) of the present invention, regardless of the length of the

15   sequence itself, may be combined with other nucleic acid sequences, including but not limited to, promoters, enhancers, polyadenylation signals, restriction enzyme sites, multiple cloning sites, coding segments, and the like, to create one or more nucleic acid construct(s). The overall length may vary considerably between nucleic acid constructs. Thus, a nucleic acid segment of almost any length may be employed, with the total length

20   preferably being limited by the ease of preparation or use in the intended recombinant nucleic acid protocol.

In a non-limiting example, one or more nucleic acid constructs may be prepared that include a contiguous stretch of nucleotides identical to or complementary to

25   SEQ ID NO:1. Such a stretch of nucleotides, or a nucleic acid construct, may be about 3, about 4, about 5, about 6, about 7, about 8,, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39 about

30   40, about 45, about 50, about 55, about 60, about 65, about 70, about 75, about 80, about

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85, about 90, about 95, about 100, about 105, about 110, about 115, about 120, about 125, about 130, about 135, about 140, about 145, about 150, about 155, about 160, about 165, about 170, about 175, about 180, about 185, about 190, about 195, about 200, about 210, about 220, about 230, about 240, about 250, about 260, about 270, about 280, about

5   290, about 300, about 310, about 320, about 330, about 340, about 350, about 360, about 370, about 380, about 390, about 400, about 410, about 420, about 430, about 440, about 450, about 460, about 470, about 480, about 490, about 500, about 510, about 520, about 530, about 540, about 550, about 560, about 570, about 580, about 590, about 600, about 610, about 618, about 650, about 700, about 750, about 1,000, about 2,000, about 3,000,

10   about 5,000, about 10,000, about 15,000, about 20,000, about 30,000, about 50,000, about 100,000, about 250,000, about 500,000, about 750,000, to about 1,000,000 nucleotides in length, as well as constructs of greater size, up to and including chromosomal sizes (including all intermediate lengths and intermediate ranges), given the advent of nucleic acids constructs such as a yeast artificial chromosome are known to

15   those of ordinary skill in the art. It will be readily understood that "intermediate lengths" and "intermediate ranges," as used herein, means any length or range including or between the quoted values (i.e., all integers including and between such values). Non-limiting examples of intermediate lengths include about 11, about 12, about 13, about 16, about 17, about 18, about 19, etc.; about 21, about 22, about 23, etc.; about 31, about 32,

20   etc.; about 51, about 52, about 53, etc.; about 101, about 102, about 103, etc.; about 151, about 152, about 153, etc.; about 1,001, about 1002, etc,; about 50,001, about 50,002, etc; about 750,001, about 750,002, etc.; about 1,000,001, about 1,000,002, etc. Non-limiting examples of intermediate ranges include about 3 to about 32, about 150 to about 500,001, about 3,032 to about 7,145, about 5,000 to about 15,000, about 20,007 to about

25   1,000,003, etc.

It is further understood that a nucleic acid sequence encoding all or a portion of an MDA-7 polypeptide may be comprised of contiguous complementary or identical nucleic acid sequences of any of the lengths described above and from SEQ ID NO:1.

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It is contemplated that the nucleic acid constructs of the present invention may encode a full-length MDA-7 or encode a truncated version of MDA-7, such that the transcript of the coding region represents the truncated version. The truncated transcript may then be translated into a truncated protein. Alternatively, a nucleic acid sequence may encode a full-length MDA-7 protein sequence, which is processed by the cellular machinery to produce a truncated MDA-7. The nucleic acid encoding a truncated transcript may contain a contiguous nucleic acid encoding a portion of mda-7 of the following lengths: about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, or 620 nucleotides, nucleosides, or base pairs. Such nucleic acid molecules may contain contiguous nucleotides of the above-lengths from SEQ ID NO:1. Thus the sequences of the present invention may contain contiguous nucleic acids that are complementary or identical to SEQ ID NO: 1, yet be less than the entire sequence of SEQ ID NO:1. For example, the sequence may contain fewer than 718 contiguous nucleic acids from SEQ ID NO:1; it may instead contain, less than 700, 690, 680, 670, 660, 650, 640, 630, 620, 610, 600, 590, 580, 570, 560, 550, 540, 530, 520, 510, 500, 490, 480, 470, 460, 450, 440, 430, 420, 410, 400, 390, 380, 370, 360, 350, 340, 330, 320, 310, 300, 290, 280, 270, 260, 250, 240, 230, 220, 210, 200, or fewer contiguous nucleotides or nucleosides from SEQ ID NO:1.

The term "a sequence essentially as set forth in SEQ ID NO:1" or "a sequence essentially as set forth in SEQ ID NO:1" means that the sequence substantially corresponds to a portion of SEQ ID NO:1 and has relatively few amino acids that are not identical to, or biologically functionally equivalent to, the amino acids of SEQ ID NO:2.

a.   Vectors and Regulatory Signals

Vectors of the present invention are designed, primarily, to transform hyperproliferative cells with a therapeutic mda-7 gene under the control of regulated eukaryotic promoters (*i.e.*, constitutive, inducible, repressable, tissue-specific). Also, the

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vectors may contain a selectable marker if, for no other reason, to facilitate their manipulation *in vitro*. However, selectable markers may play an important role in producing recombinant cells.

5   Tables 1 and 2, below, list a variety of regulatory signals for use according to the present invention.

Table 1 - Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987; Karin ®, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Fonta *et al.*, 1985; Sakai *et al.*, 1986
ß-Interferon	poly(rI)X poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 <u>E2</u>	ElA	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b
SV40	Phorbol Ester (TFA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	Hug et a., 1988
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	ElA, SV40 Large T Antigen	Taylor *et al.*, 1989; Taylor and Kingston, 1990a,b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	PMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone α Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

Table 2 - Other Promoter/Enhancer Elements

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1988; Kiledjian *et al.*, 1988; Porton *et al.*, 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987, Winoto and Baltimore, 1989; Redondo *et al.*, 1990
HLA DQ α and DQ β	Sullivan and Peterlin, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1985
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II	Koch *et al.*, 1989
MHC Class II HLA-DRα	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*, 1989
Muscle Creatine Kinase	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989a
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Omitz *et al.*, 1987
Metallothionein	Karin *et al.*, 1987; Culotta and Hamer, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987
Albumin Gene	Pinkert *et al.*, 1987, Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989
γ-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β-Globin	Trudel and Constantini, 1987
c-fos	Cohen *et al.*, 1987
c-HA-ras	Triesman, 1986; Deschamps *et al.*, 1985

Promoter/Enhancer	References
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsch *et al.*, 1990
a_1-antitrypsin	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987 Schaffner *et al.*, 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; deVilliers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a,b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987, Stephens and Hentschel, 1987; Glue *et al.*, 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988

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Promoter/Enhancer	References
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rowen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989
Cytomegalovirus	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

The promoters and enhancers that control the transcription of protein encoding genes in eukaryotic cells are composed of multiple genetic elements. The cellular machinery is able to gather and integrate the regulatory information conveyed by each element, allowing different genes to evolve distinct, often complex patterns of transcriptional regulation. A promoter used in the context of the present invention includes constitutive, inducible, and tissue-specific promoters.

The term "promoter" will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

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Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements

5   downstream of the start site as well. The spacing between elements is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

10

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA

15   with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need

20   not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities.

25   Promoters and enhancers have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional

30   machinery in fundamentally the same way.

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Preferred for use in the present invention is the cytomegalovirus (CMV) promoter. This promoter is commercially available from Invitrogen in the vector pcDNAIII, which is preferred for use in the present invention. Also contemplated as useful in the present invention are the dectin-1 and dectin-2 promoters. Additional viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the present invention are listed in Tables 1 and 2. Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of structural genes encoding oligosaccharide processing enzymes, protein folding accessory proteins, selectable marker proteins or a heterologous protein of interest. Alternatively, a tissue-specific promoter for cancer gene therapy (Table 3) or the targeting of tumors (Table 4) may be employed with the nucleic acid molecules of the present invention.

Table 3: Candidate tissue-specific promoters for cancer gene therapy

Tissue-specific promoter	Cancers in which promoter is active	Normal cells in which promoter is active
Carcinoembryonic antigen (CEA)*	Most colorectal carcinomas; 50% of lung carcinomas; 40-50% of gastric carcinomas; most pancreatic carcinomas; many breast carcinomas	Colonic mucosa; gastric mucosa; lung epithelia; eccrine sweat glands; cells in testes
Prostate-specific antigen (PSA) ··	Most prostate carcinomas	Prostate epithelium
Vasoactive intestinal peptide (VIP)	Majority of non-small cell lung cancers	Neurons; lymphocytes; mast cells; eosinophils
Surfactant protein A (SP-A) cells	Many lung adenocarcinomas	Type II pneumocytes; Clara
Human achaete-scute homolog (hASH)	Most small cell lung cancers	Neuroendocrine cells in lung
Mucin-1 (MUC1)** breast	Most adenocarcinomas (originating from any tissue)	GFandular epithelial cells in and in respiratory, gastrointestinal, and genitourinary tracts
Alpha-fetoprotein	Most hepatocellular carcinomas; possibly many testicular cancers	Hepatocytes (under certain conditions); testis
Albumin	Most hepatocellular carcinomas	Hepatocytes
Tyrosinase	Most melanomas	Melanocytes; astrocytes; Schwann cells; some neurons
Tyrosine-binding protein (TRP)	Most melanomas	Melanocytes; astrncytes, Schwann cells; some neurons
Keratin 14	Prosumably many squamous cei[1] carcinomas (eg: Head and neck cancers)	Keratinocytes
EBV LD-2	Many squamous cell carcinomas of head and neck	Keratinocytes of upper digestive tract
Glial fibrillary acidic protein (GFAP)	Many astrocytomas	Astrocytes
Myelin basic protein (MBP)	Many gliomas	Oligodendrocytes
Testis-specific angiotensin-converting enzyme (Testis-specific ACE)	Possibly many testicular cancers	Spermatazoa
Osteocalcin	Possibly many osteosarcomas	Osteoblasts

Table 4: Candidate promoters for use with a tissue-specific targeting of tumors

	Promoter	Cancers in which Promoter is active	Normal cells in which Promoter is active
5			
	E2F-regulated promoter	Almost all cancers	Proliferating cells
	HLA-G	Many colorectal carcinomas; many melanomas; possibly many other cancers	Lymphocytes; monocytes; spermatocytes; trophoblast
10	FasL neurons;	Most melanomas; many pancreatic carcinomas; most astrocytomas;	Activated leukocytes: endothelial cells;
	keratinocytes;	possibly many other cancers	cells in immunoprivileged
15			tissues; some cells in lungs, ovaries, liver, and prostate
	Myc-regulated promoter some	Most lung carcinomas (both small	Proliferating cells (only
		cell and non-small cell); most	cell-types): mammary
20	epithelial	colorectal carcinomas	cells (including non-
	proliferating)		
	MAGE-1	Many melanomas; some non-small cell lung carcinomas; some breast carcinomas	Testis
25			
	VEGF	70% of all cancers (constitutive overexpression in many cancers)	Cells at sites of neovascularization (but
	unlike in		
30	transient,		tumors, expression is
			less strong, and never constitutive)
	bFGF	Presumably many different cancers, since bFGF expression is induced by ischemic conditions	cells at sites of ischemia (but unlike tumors, expression is transient, less strong, and
35	never		
			constitutive)
	COX-2 inflammation	Most colorectal carcinomas; many	Cells at sites of
40		lung carcinomas; possibly many other cancers	
	IL-10	Most colorectal carcinomas; many lung carcinomas; many squamous cell carcinomas of head and neck;	Leukocytes
45		possibly many other cancers	
	GRP78/BiP	Presumably many different cancers, since GRP7S expression is induced by tumor-specific conditions	Cells at sites of ischemia
50	CArG elements from Egr-1	Induced by ionization radiation, so conceivably most tumors upon irradiation	Cells exposed to ionizing radiation; leukocytes

Another signal that may prove useful is a polyadenylation signal (hGH, BGH, SV40). The use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′–methylated cap-dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well as an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

In any event, it will be understood that promoters are DNA elements which when positioned functionally upstream of a gene leads to the expression of that gene. Most transgene constructs of the present invention are functionally positioned downstream of a promoter element.

b.   Gene Transfer

i.   Viral Transformation

a)   Adenoviral Infection

One method for delivery of the recombinant DNA involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a recombinant gene construct that has been cloned therein.

The vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization or adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host

5   cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification.

Adenovirus is particularly suitable for use as a gene transfer vector because of its

10   mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes

15   proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant

20   processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

25   In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

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Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3, or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone.

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Racher *et al.* (1995) have disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume)

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and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

The adenovirus vector may be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the transforming construct at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.* (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus growth and manipulation is known to those of skill in the art, and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

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Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Animal studies have suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

b)   Retroviral Infection

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the

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RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

Concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, packaging cell lines are available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

c) AAV Infection

Adeno-associated virus (AAV) is an attractive vector system for use in the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells in tissue culture (Muzyczka, 1992). AAV has a broad host range for infectivity (Tratschin, *et al.*, 1984; Laughlin, *et al.*, 1986; Lebkowski, *et al.*, 1988; McLaughlin, *et al.*, 1988), which means it is applicable for use with the present invention. Details concerning the generation and use of rAAV vectors are described in U.S. Patent No. 5,139,941 and U.S. Patent No. 4,797,368, each incorporated herein by reference.

Studies demonstrating the use of AAV in gene delivery include LaFace *et al.* (1988); Zhou *et al.* (1993); Flotte *et al.* (1993); and Walsh *et al.* (1994). Recombinant AAV vectors have been used successfully for *in vitro* and *in vivo* transduction of marker genes (Kaplitt *et al.*, 1994; Lebkowski *et al.*, 1988; Samulski *et al.*, 1989; Shelling and Smith, 1994; Yoder *et al.*, 1994; Zhou *et al.*, 1994; Hermonat and Muzyczka, 1984; Tratschin *et al.*, 1985; McLaughlin *et al.*, 1988) and genes involved in human diseases

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(Flotte *et al.*, 1992; Luo *et al.*, 1994; Ohi *et al.*, 1990; Walsh *et al.*, 1994; Wei *et al.*, 1994). Recently, an AAV vector has been approved for phase I human trials for the treatment of cystic fibrosis.

5   AAV is a dependent parvovirus in that it requires coinfection with another virus (either adenovirus or a member of the herpes virus family) to undergo a productive infection in cultured cells (Muzyczka, 1992). In the absence of coinfection with helper virus, the wild-type AAV genome integrates through its ends into human chromosome 19 where it resides in a latent state as a provirus (Kotin *et al.*, 1990; Samulski *et al.*, 1991).
10   rAAV, however, is not restricted to chromosome 19 for integration unless the AAV Rep protein is also expressed (Shelling and Smith, 1994). When a cell carrying an AAV provirus is superinfected with a helper virus, the AAV genome is "rescued" from the chromosome or from a recombinant plasmid, and a normal productive infection is established (Samulski *et al.*, 1989; McLaughlin *et al.*, 1988; Kotin *et al.*, 1990;
15   Muzyczka, 1992).

          Typically, recombinant AAV (rAAV) virus is made by cotransfecting a plasmid containing the gene of interest flanked by the two AAV terminal repeats (McLaughlin *et al.*, 1988; Samulski *et al.*, 1989; each incorporated herein by reference) and an
20   expression plasmid containing the wild-type AAV coding sequences without the terminal repeats, for example pIM45 (McCarty *et al.*, 1991; incorporated herein by reference). The cells are also infected or transfected with adenovirus or plasmids carrying the adenovirus genes required for AAV helper function. rAAV virus stocks made in such fashion are contaminated with adenovirus which must be physically separated from the
25   rAAV particles (for example, by cesium chloride density centrifugation). Alternatively, adenovirus vectors containing the AAV coding regions or cell lines containing the AAV coding regions and some or all of the adenovirus helper genes could be used (Yang *et al.*, 1994; Clark *et al.*, 1995). Cell lines carrying the rAAV DNA as an integrated provirus can also be used (Flotte *et al.*, 1995).

30

d)   Other Viral Vectors

Other viral vectors may be employed as constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

A molecularly cloned strain of Venezuelan equine encephalitis (VEE) virus has been genetically refined as a replication competent vaccine vector for the expression of heterologous viral proteins (Davis *et al.*, 1996). Studies have demonstrated that VEE infection stimulates potent CTL responses and has been sugested that VEE may be an extremely useful vector for immunizations (Caley *et al.*, 1997). It is contemplated in the present invention, that VEE virus may be useful in targeting dendritic cells.

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. Chang *et al.* recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

In still further embodiments of the present invention, the nucleic acid encoding human mda-7 is housed within an infective virus that has been engineered to express a specific binding ligand. The virus particle will thus bind specifically to the cognate receptors of the target cell and deliver the contents to the cell. A novel approach designed

to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification can permit the specific infection of hepatocytes via sialoglycoprotein receptors.

For example, targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

ii.   Non-Viral Delivery

In addition to viral delivery of the nucleic acid encoding full length or truncated MDA-7 protein, the following are additional methods of recombinant gene delivery to a given host cell and are thus considered in the present invention.

a)   Electroporation

In certain preferred embodiments of the present invention, the gene construct is introduced into target hyperproliferative cells via electroporation. Electroporation involves the exposure of cells (or tissues) and DNA (or a DNA complex) to a high-voltage electric discharge.

Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter *et al.*, 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa *et al.*, 1986) in this manner.

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It is contemplated that electroporation conditions for hyperproliferative cells from different sources may be optimized. One may particularly wish to optimize such parameters as the voltage, the capacitance, the time and the electroporation media composition. The execution of other routine adjustments will be known to those of skill in the art. *See e.g.,* Hoffman, 1999; Heller *et al.,* 1996.

b) Particle Bombardment

Another embodiment of the invention for transferring a naked DNA construct into cells involves particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.,* 1987). The microprojectiles used have consisted of biologically inert substances such as tungsten, platinum, or gold beads.

It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using particle bombardment. It is contemplated that particles may contain DNA rather than be coated with DNA. Hence it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.,* 1990). Another method involves the use of a Biolistic Particle Delivery System, which can be used to propel particles coated with DNA through a screen, such as stainless steel or Nytex screen, onto a filter surface covered with cells in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectile aggregates and may contribute to a higher frequency of transformation by reducing the damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters, or alternatively on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between the acceleration device and the cells to be

5   bombarded.

In bombardment transformation, one may optimize the prebombardment culturing conditions and the bombardment parameters to yield the maximum numbers of stable transformants. Both the physical and biological parameters for bombardment are

10   important in this technology. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity or either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, and also the nature of the

15   transforming DNA, such as linearized DNA or intact supercoiled plasmids. Recently, results from a clinical trial evaluating utility of this delivery system for vaccination was published. The study was designed to determine the safety and immunogenicity in volunteers of a DNA vaccine consisting of a plasmid encoding hepatitis B surface antigen delivered by the PowderJect XR1 gene delivery system into human skin (Tacket *et al.*,

20   1999).

Accordingly, it is contemplated that one may wish to adjust various bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance

25   and helium pressure. One also may optimize the trauma reduction factors by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be

30   known to those of skill in the art.

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c) Calcium Phosphate Co-Precipitation or DEAE-Dextran Treatment

In other embodiments of the present invention, the transgenic construct is introduced to the cells using calcium phosphate co-precipitation. Mouse primordial germ cells have been transfected with the SV40 large T antigen, with excellent results (Watanabe *et al.*, 1997). Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe *et al.*, 1990).

In another embodiment, the expression construct is delivered into the cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal, 1985).

d) Direct Microinjection or Sonication Loading

Further embodiments of the present invention include the introduction of the nucleic acid construct by direct microinjection or sonication loading. Direct microinjection has been used to introduce nucleic acid constructs into *Xenopus* oocytes (Harland and Weintraub, 1985), and LTK⁻ fibroblasts have been transfected with the thymidine kinase gene by sonication loading (Fechheimer *et al.*, 1987).

e) Lipid-Mediated Transformation

In a further embodiment of the invention, the gene construct may be entrapped in a liposome or lipid formulation. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water

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and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated is a gene construct complexed with Lipofectamine (Gibco BRL).

Lipid-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has
5   been very successful (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987). Wong *et al.* (1980) demonstrated the feasibility of lipid-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells.

Lipid based non-viral formulations provide an alternative to adenoviral gene
10   therapies. Although many cell culture studies have documented lipid based non-viral gene transfer, systemic gene delivery via lipid based formulations has been limited. A major limitation of non-viral lipid based gene delivery is the toxicity of the cationic lipids that comprise the non-viral delivery vehicle. The *in vivo* toxicity of liposomes partially explains the discrepancy between *in vitro* and *in vivo* gene transfer results. Another
15   factor contributing to this contradictory data is the difference in lipid vehicle stability in the presence and absence of serum proteins. The interaction between lipid vehicles and serum proteins has a dramatic impact on the stability characteristics of lipid vehicles (Yang and Huang, 1997). Cationic lipids attract and bind negatively charged serum proteins. Lipid vehicles associated with serum proteins are either dissolved or taken up
20   by macrophages leading to their removal from circulation. Current *in vivo* lipid delivery methods use subcutaneous, intradermal, intratumoral, or intracranial injection to avoid the toxicity and stability problems associated with cationic lipids in the circulation. The interaction of lipid vehicles and plasma proteins is responsible for the disparity between the efficiency of *in vitro* (Felgner *et al.*, 1987) and *in vivo* gene transfer (Zhu *et al.*, 1993;
25   Philip *et al.*, 1993; Solodin *et al.*, 1995; Liu *et al.*, 1995; Thierry *et al.*, 1995; Tsukamoto *et al.*, 1995; Aksentijevich *et al.*, 1996).

Recent advances in lipid formulations have improved the efficiency of gene transfer *in vivo* (Templeton *et al.* 1997; WO 98/07408). A novel lipid formulation
30   composed of an equimolar ratio of 1,2-bis(oleoyloxy)-3-(trimethyl ammonio)propane

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(DOTAP) and cholesterol significantly enhances systemic *in vivo* gene transfer, approximately 150 fold. The DOTAP:cholesterol lipid formulation forms unique structure termed a "sandwich liposome". This formulation is reported to "sandwich" DNA between an invaginated bi-layer or 'vase' structure. Beneficial characteristics of

5   these lipid structures include a positive p, colloidal stabilization by cholesterol, two dimensional DNA packing and increased serum stability. Patent Application Nos. 60/135,818 and 60/133,116 discuss formulations that may be used with the present invention and are incorporated herein by reference.

10   The production of lipid formulations often is accomplished by sonication or serial extrusion of liposomal mixtures after (I) reverse phase evaporation (II) dehydration-rehydration (III) detergent dialysis and (IV) thin film hydration. Once manufactured, lipid structures can be used to encapsulate compounds that are toxic (chemotherapeutics) or labile (nucleic acids) when in circulation. Lipid encapsulation has resulted in a lower

15   toxicity and a longer serum half-life for such compounds (Gabizon *et al.*, 1990). Numerous disease treatments are using lipid based gene transfer strategies to enhance conventional or establish novel therapies, in particular therapies for treating hyperproliferative diseases.

20   In certain embodiments of the invention, the lipid vehicle may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of lipid-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the lipid vehicle may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further

25   embodiments, the lipid vehicle may be complexed or employed in conjunction with both HVJ and HMG-1.

A contemplated method for commercial scale preparation of a lipid composition is, generally speaking, mixing of the components, drying the mixture, re-hydrating the

30   mixture, dispersing the mixture, extruding the lipid composition through filters of

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decreasing pore size and mixing of the lipid composition with a therapeutic agent to form a lipoplex. The following provides additional information about manufacturing and formulating a lipoplex for use in the delivery of an mda-7 polynucleotide to a cell.

5   Powdered components are weighed, mixed, and dissolved in an acceptable solvent, such as tertiary butanol, chloroform, methanol, ethylene chloride, ethanol, or mixtures of these solvents. It is contemplated that any two of the solvent are present in a ration of about 1:1000, 1:500,1:100, 1:50, 1:25, 1:10, 1:5 or 1:1. Solublization with tertiary butanol may be employed, due to the carcinogenic properties of chloroform.

10   Although, chloroform can be used if steps are taken to limit the residual chloroform present in the lipid composition to acceptable levels. It is envisioned that other lipophilic solvents may be used for the solubilization of the lipid components. The lipid mixture may be solubilized in *tert*-butanol at a temperature of about 0°C, 2°C, 4°C, 6°C, 8°C, 10°C, 12°C, 14°C, 16°C, 18°C, 20°C, 22°C, 24°C, 26°C, 28°C, 30°C, 32°C, 34°C, 36°C,

15   38°C, 40°C, 42°C, 44°C, 46°C, 48°C, 50°C, 52°C, 54°C, 56°C, 58°C, 60°C, 62°C, 64°C, 66°C, 68°C, 70°C, 72°C, 74°C, 76°C, 78°C, 80°C, 82°C, 84°C, 86°C, 88°C, or 90°C. A range of temperatures, such as 5°C to 80°C, 10°C to 70°C, 20°C to 60°C, and 30°C to 50°C, is also contemplated for use with the present invention.

20   Following preparation of the lipid composition a lipid complex is formulated by combining the lipid composition and a therapeutic agent in a pharmaceutically acceptable carrier solution by rapid mixing. Rapid mixing can be done by using a T-mixing apparatus or ethanol injection of the therapeutic agent. In certain embodiments a DOTAP:Cholesterol lipid composition is prepared by the methods described and

25   combined with a polynucleotide that encodes for a therapeutic protein. The lipid composition is provided in an amount to encapsulate the polynucleotide and result in a colloidal suspension of the lipoplex.

DOTAP (cationic lipid) may be mixed with cholesterol at equimolar concentrations. This mixture of powdered lipids is then dissolved with tert-butanol, the solution dried to a thin film and the film hydrated in water containing 5% dextrose (w/v) to give a final concentration of about 20 mM DOTAP and about 20 mM cholesterol. The

5   hydrated lipid film is rotated in a heated water bath for about 45 minutes, then at about 35°C for an additional 10 minutes and left standing at room temperature overnight. The following day the mixture is sonicated for 5 minutes at about 50°C. The sonicated mixture is heated for 10 minutes at about 50°C. This mixture is sequentially extruded through filters of decreasing pore size (1 μm, 0.45 μm, 0.2 μm, 0.1 μm). This lipid

10   composition is then mixed with DNA to produce a lipid complex.

A lipid composition may comprise DOTAP and cholesterol, a cholesterol derivative or a cholesterol mixture and a polynucleotide encoding a therapeutic protein, such as MDA-7, antisense RNA or ribozyme for delivery into disease cells or into cells

15   near the disease cells. The treatment of a disease, in one embodiment, involves the intravenous administration of a polynucleotide lipoplex to a patient, which subsequently express a therapeutic encoded by the polynucleotide. The lipoplex treatment of the patient delivers the polynucloetide to normal and hyperproliferative cells that express the therapeutic, resulting in the inhibition or destruction of the hyperproliferative cells.

20

The initial lipid mixture will preferably be of powdered lipid components that can be weighed and mixed to appropriate molar ratios. The components can be anionic lipids, cationic lipids, neutral lipids, sterols, and/or other hydrophobic molecules in ratios necessary to produce the desired characteristics of the final lipid composition or complex.

25   The actual composition of the lipid mixture will be determined by the properties required for efficient delivery of the agent(s) to the desired target cells by the desired means of administration, described in detail below. Components of the composition can be mixed to provide various molar ratios. The molar concentrations of any component of the lipid composition can be from about 0.5mM, 1mM, 2mM, 3mM, 4mM, 5mM, 6mM, 7mM,

30   8mM, 9mM, 10mM, 15mM, 20mM, 25mM, 30mM, 35mM, 40mM, 45mM, and 50mM.

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The molar ratio of any two of the components can be from about 1:100. 1:50, 1:25, 1:20:1:18, 1:16, 1:15, 1:14, 1:13, 1:12, 1:11, 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 1:0.5, 1:0.25, 1:0.1, 1:0.05, or 1:0.01.

5   The lipid compositions are capable of carrying biologically active agents. The lipid composition can sequester toxic compounds to reduce free concentrations in the serum, protect compounds from degradative agents in the body, and/or mask antigenic components to reduce the immunogenicity of the agent. For example, DOTP:Cholesterol lipid compositions complex with polynucleotides such that the polynucleotides are 10   sequestered and resistant to degradation in the serum. The lipid composition may be complexed with a variety of therapeutic agents, including but not limited to polynucleotides, proteins, peptides, chemotherapeutics, small molecule, peptonucleotides, and carbohydrate therapeutics.

15   A preferred lipid composition is DOTAP and cholesterol or a cholesterol derivative. The ratio of DOTAP to cholesterol, cholesterol derivative or cholesterol mixture may about 4:1 to 1:4, 3:1 to 1:3, more preferably 2:1 to 1:2, or 1:1. The DOTAP or cholesterol concentrations may be between about 1 to 8 mM, 2 to 7 mM, 3 to 6 mM, or 4 to 5 mM. Cholesterol derivatives may be readily substituted for the cholesterol or 20   mixed with the cholesterol in the present invention. A number of cholesterol derivatives are known to the skilled artisan. Cholesterol acetate and cholesterol oleate may be used. Polynucleotides are preferably added to the liposomes at a concentration of 20 to 200 μg per 200 μL final volume.

25   The lipoplex is prepared by diluting a given polynucleotide and lipid composition in 5% dextrose in water to obtain an appropriate concentration of nucleic acid and lipids. Equal volumes of nucleic acid and lipids, at a concentration to obtain 100 μg of nucleic acid / 5mM lipids / 100 μl, is mixed by adding the nucleic acid rapidly to the surface of the lipid composition by rapid mixing or with an impinging jet.

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3. Pharmaceutical Formulations and Delivery

In a preferred embodiment of the present invention, a method of treatment for a hyperproliferative disease by the delivery of an expression construct encoding either a full length or truncated human mda-7 protein is contemplated. Hyperproliferative diseases that are most likely to be treated in the present invention are those that result from mutations in an oncogene and/ or the reduced expression of a wild-type protein in the hyperproliferative cells. Examples of hyperproliferative diseases contemplated for treatment include lung cancer, head and neck cancer, breast cancer, pancreatic cancer, prostate cancer, renal cancer, bone cancer, testicular cancer, cervical cancer, gastrointestinal cancer, lymphomas, pre-neoplastic lesions in the lung, colon cancer, melanoma, bladder cancer and any other hyperproliferative diseases that may be treated by administering a nucleic acid encoding either a full length or truncated human mda-7 protein.

An effective amount of the pharmaceutical composition, generally, is defined as that amount sufficient to detectably and repeatedly to ameliorate, reduce, minimize or limit the extent of the disease or its symptoms. More rigorous definitions may apply, including elimination, eradication or cure of disease.

Preferably, patients will have adequate bone marrow function (defined as a peripheral absolute granulocyte count of > 2,000 / mm^3 and a platelet count of 100,000 / mm^3), adequate liver function (bilirubin < 1.5 mg / dl) and adequate renal function (creatinine < 1.5 mg / dl).

a. Administration

To kill cells, inhibit cell growth, inhibit metastasis, decrease tumor or tissue size and otherwise reverse or reduce the malignant phenotype of tumor cells, using the methods and compositions of the present invention, one would generally contact a hyperproliferative cell with the therapeutic expression construct. The routes of administration will vary, naturally, with the location and nature of the lesion, and include,

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e.g., intradermal, transdermal, parenteral, intravenous, intramuscular, intranasal, subcutaneous, percutaneous, intratracheal, intraperitoneal, intratumoral, perfusion, lavage, direct injection, and oral administration and formulation.

5   Intratumoral injection, or injection into the tumor vasculature is specifically contemplated for discrete, solid, accessible tumors. Local, regional or systemic administration also may be appropriate. For tumors of >4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of <4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple injections delivered as

10   single dose comprise about 0.1 to about 0.5 ml volumes. The viral particles may advantageously be contacted by administering multiple injections to the tumor, spaced at approximately 1 cm intervals.

        In the case of surgical intervention, the present invention may be used

15   preoperatively, to render an inoperable tumor subject to resection. Alternatively, the present invention may be used at the time of surgery, and/or thereafter, to treat residual or metastatic disease. For example, a resected tumor bed may be injected or perfused with a formulation comprising mda-7 or an mda-7-encoding construct. The perfusion may be continued post-resection, for example, by leaving a catheter implanted at the site of the

20   surgery. Periodic post-surgical treatment also is envisioned.

        Continuous administration also may be applied where appropriate, for example, where a tumor is excised and the tumor bed is treated to eliminate residual, microscopic disease. Delivery *via* syringe or catherization is preferred. Such continuous perfusion

25   may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment. Generally, the dose of the therapeutic composition *via* continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs. It is further contemplated that limb perfusion

may be used to administer therapeutic compositions of the present invention, particularly in the treatment of melanomas and sarcomas.

Treatment regimens may vary as well, and often depend on tumor type, tumor location, disease progression, and health and age of the patient. Obviously, certain types of tumor will require more aggressive treatment, while at the same time, certain patients cannot tolerate more taxing protocols. The clinician will be best suited to make such decisions based on the known efficacy and toxicity (if any) of the therapeutic formulations.

In certain embodiments, the tumor being treated may not, at least initially, be resectable. Treatments with therapeutic viral constructs may increase the resectability of the tumor due to shrinkage at the margins or by elimination of certain particularly invasive portions. Following treatments, resection may be possible. Additional treatments subsequent to resection will serve to eliminate microscopic residual disease at the tumor site.

A typical course of treatment, for a primary tumor or a post-excision tumor bed, will involve multiple doses. Typical primary tumor treatment involves a 6 dose application over a two-week period. The two-week regimen may be repeated one, two, three, four, five, six or more times. During a course of treatment, the need to complete the planned dosings may be re-evaluated.

The treatments may include various "unit doses." Unit dose is defined as containing a predetermined-quantity of the therapeutic composition. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. A unit dose need not be administered as a single injection but may comprise continuous infusion over a set period of time. Unit dose of the present invention may conveniently be described in terms of plaque forming units (pfu) for a viral construct. Unit doses range from 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13} pfu and higher.

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Alternatively, depending on the kind of virus and the titer attainable, one will deliver 1 to 100, 10 to 50, 100-1000, or up to about 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8, 1×10^9, 1×10^{10}, 1×10^{11}, 1×10^{12}, 1×10^{13}, 1×10^{14}, or 1×10^{15} or higher infectious viral particles (vp) to the patient or to the patient's cells.

5

b.   Injectable Compositions and Formulations

The preferred method for the delivery of an expression construct encoding either a full length or truncated human MDA-7 protein to hyperproliferative cells in the present invention is via intratumoral injection. However, the pharmaceutical compositions

10   disclosed herein may alternatively be administered parenterally, intravenously, intradermally, intramuscularly, transdermally or even intraperitoneally as described in U.S. Patent 5,543,158; U.S. Patent 5,641,515 and U.S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety).

15   Injection of nucleic acid constructs may be delivered by syringe or any other method used for injection of a solution, as long as the expression construct can pass through the particular gauge of needle required for injection. A novel needleless injection system has recently been described (U.S. Patent 5,846,233) having a nozzle defining an ampule chamber for holding the solution and an energy device for pushing the solution

20   out of the nozzle to the site of delivery. A syringe system has also been described for use in gene therapy that permits multiple injections of predetermined quantities of a solution precisely at any depth (U.S. Patent 5,846,225).

Solutions of the active compounds as free base or pharmacologically acceptable

25   salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile

30   aqueous solutions or dispersions and sterile powders for the extemporaneous preparation

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of sterile injectable solutions or dispersions (U.S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating

5   action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of

10   dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in

15   the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with

20   sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, intratumoral and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of

25   hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration,

preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vaccuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its

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use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" or "pharmacologically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared.

4. Combination Treatments

In order to increase the effectiveness of a full-length, substantially full-length, or truncated mda-7 polypeptide, or expression construct coding therefor, it may be desirable to combine these compositions with other agents effective in the treatment of hyperproliferative disease, such as anti-cancer agents, or with surgery. An "anti-cancer" agent is capable of negatively affecting cancer in a subject, for example, by killing cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or cancer cells, promoting an immune response against cancer cells or a tumor, preventing or inhibiting the progression of cancer, or increasing the lifespan of a subject with cancer. Anti-cancer agents include biological agents (biotherapy), chemotherapy agents, and radiotherapy agents. More generally, these other compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the expression construct and the agent(s) or multiple factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the second agent(s).

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Tumor cell resistance to chemotherapy and radiotherapy agents represents a major problem in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy by combining it with gene therapy. For

5   example, the herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, et al., 1992). In the context of the present invention, it is contemplated that mda-7 gene therapy could be used similarly in conjunction with chemotherapeutic, radiotherapeutic, immunotherapeutic or other biological intervention,

10   in addition to other pro-apoptotic or cell cycle regulating agents.

Alternatively, the gene therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and expression construct are applied separately to the cell, one would generally ensure that a

15   significant period of time did not expire between the time of each delivery, such that the agent and expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one may contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for

20   treatment significantly, however, where several d (2, 3, 4, 5, 6 or 7) to several wk (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

Various combinations may be employed, gene therapy is "A" and the secondary agent, such as radio- or chemotherapy, is "B":

25

A/B/A   B/A/B   B/B/A   A/A/B   A/B/B   B/A/A   A/B/B/B   B/A/B/B

B/B/B/A   B/B/A/B   A/A/B/B   A/B/A/B   A/B/B/A   B/B/A/A

30   B/A/B/A   B/A/A/B   A/A/A/B   B/A/A/A   A/B/A/A   A/A/B/A

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Administration of the therapeutic expression constructs of the present invention to a patient will follow general protocols for the administration of chemotherapeutics, taking into account the toxicity, if any, of the vector. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the described hyperproliferative cell therapy.

a.   Chemotherapy

Cancer therapies also include a variety of combination therapies with both chemical and radiation based treatments. Combination chemotherapies include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, raloxifene, estrogen receptor binding agents, taxol, gemcitabien, navelbine, farnesyl-protein transferase inhibitors, transplatinum, 5-fluorouracil, vincristine, vinblastine and methotrexate, Temazolomide (an aqueous form of DTIC), or any analog or derivative variant of the foregoing. The combination of chemotherapy with biological therapy is known as biochemotherapy.

b.   Radiotherapy

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely,

and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic construct and a chemotherapeutic or radiotherapeutic agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or stasis, both agents are delivered to a cell in a combined amount effective to kill the cell or prevent it from dividing.

c.   Immunotherapy

Immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, *etc.*) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells. Mda-7 gene transfer to tumor cells causes tumor cell death and apoptosis. The apoptotic tumor cells are scavenged by reticuloendothelial cells including dendritic cells and macrophages and presented to the immune system to generate anti-tumor immunity (Rovere *et al.*, 1999; Steinman *et al.*, 1999). The soluble form of MDA-7 protein has cytokine-like structure and activities, such as activation of immune cells. The combination of therapeutic modalities, i.e., direct cytotoxic activity and immune activation by MDA-7 would provide therapeutic benefit in the treatment of cancer.

Immunotherapy could also be used as part of a combined therapy, in conjunction with Ad-mda7 gene therapy. The general approach for combined therapy is discussed below. In one aspect of immunotherapy, the tumor cell must bear some marker that is

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amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72,

5   HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, *erb* B and p155. An alternative aspect of immunotherapy is to combine pro-apoptotic effect, mediated by Ad-mda7 treatment with immune stimulatory effects. The latter may be inherent in the soluble MDA-7 protein. However, alternate immune stimulating molecules also exist including: cytokines such as IL-2, IL-4, IL-12, GM-CSF, gamma-

10   IFN, chemokines such as MIP-1, MCP-1, IL-8 and growth factors such as FLT3 ligand. Combining immune stimulating molecules, either as proteins or using gene delivery in combination with Ad-mda7 will enhance anti-tumor effects (Ju *et al.*, 2000).

As discussed earlier, examples of immunotherapies currently under investigation

15   or in use are immune adjuvants (*e.g.*, *Mycobacterium bovis*, *Plasmodium falciparum*, dinitrochlorobenzene and aromatic compounds) (U.S. Patent 5,801,005; U.S. Patent 5,739,169; Hui and Hashimoto, 1998; Christodoulides *et al.*, 1998), cytokine therapy (*e.g.*, interferons α, β and γ; IL-1, GM-CSF and TNF) (Bukowski *et al.*, 1998; Davidson *et al.*, 1998; Hellstrand *et al.*, 1998) gene therapy (*e.g.*, TNF, IL-1, IL-2, p53) (Qin *et al.*,

20   1998; Austin-Ward and Villaseca, 1998; U.S. Patent 5,830,880 and U.S. Patent 5,846,945) and monoclonal antibodies (*e.g.*, anti-ganglioside GM2, anti-HER-2, anti-p185) (Pietras *et al.*, 1998; Hanibuchi *et al.*, 1998; U.S. Patent 5,824,311). Herceptin (trastuzumab) is a chimeric (mouse-human) monoclonal antibody that blocks the HER2-neu receptor. It possesses anti-tumor activity and has been approved for use in the

25   treatment of malignant tumors (Dillman, 1999). Combination therapy of cancer with herceptin and chemotherapy has been shown to be more effective than the individual therapies. Thus, it is contemplated that one or more anti-cancer therapies may be employed with the Ad-mda7 therapy described herein.

i.   Passive Immunotherapy

A number of different approaches for passive immunotherapy of cancer exist. They may be broadly categorized into the following: injection of antibodies alone; injection of antibodies coupled to toxins or chemotherapeutic agents; injection of antibodies coupled to radioactive isotopes; injection of anti-idiotype antibodies; and finally, purging of tumor cells in bone marrow.

Preferably, human monoclonal antibodies are employed in passive immunotherapy, as they produce few or no side effects in the patient. However, their application is somewhat limited by their scarcity and have so far only been administered intralesionally. Human monoclonal antibodies to ganglioside antigens have been administered intralesionally to patients suffering from cutaneous recurrent melanoma (Irie & Morton, 1986). Regression was observed in six out of ten patients, following, daily or weekly, intralesional injections. In another study, moderate success was achieved from intralesional injections of two human monoclonal antibodies (Irie *et al.*, 1989).

It may be favorable to administer more than one monoclonal antibody directed against two different antigens or even antibodies with multiple antigen specificity. Treatment protocols also may include administration of lymphokines or other immune enhancers as described by Bajorin *et al.* (1988). The development of human monoclonal antibodies is described in further detail elsewhere in the specification.

ii.   Active Immunotherapy

In active immunotherapy, an antigenic peptide, polypeptide or protein, or an autologous or allogenic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant (Ravindranath & Morton, 1991; Morton & Ravindranath, 1996; Morton *et al.*, 1992; Mitchell *et al.*, 1990; Mitchell *et al.*, 1993). In melanoma immunotherapy, those patients who elicit high IgM response often survive better than those who elicit no or low IgM antibodies (Morton *et al.*, 1992). IgM

antibodies are often transient antibodies and the exception to the rule appears to be anti-ganglioside or anticarbohydrate antibodies.

iii. Adoptive Immunotherapy

In adoptive immunotherapy, the patient's circulating lymphocytes, or tumor infiltrated lymphocytes, are isolated *in vitro*, activated by lymphokines such as IL-2 or transduced with genes for tumor necrosis, and readministered (Rosenberg *et al.*, 1988; 1989). To achieve this, one would administer to an animal, or human patient, an immunologically effective amount of activated lymphocytes in combination with an adjuvant-incorporated anigenic peptide composition as described herein. The activated lymphocytes will most preferably be the patient's own cells that were earlier isolated from a blood or tumor sample and activated (or "expanded") *in vitro*. This form of immunotherapy has produced several cases of regression of melanoma and renal carcinoma, but the percentage of responders were few compared to those who did not respond.

d. Genes

In yet another embodiment, the secondary treatment is a secondary gene therapy in which a second therapeutic polynucleotide is administered before, after, or at the same time a first therapeutic polynucleotide encoding all of part of an MDA-7 polypeptide. Delivery of a vector encoding either a full length or truncated MDA-7 in conjuction with a second vector encoding one of the following gene products will have a combined anti-hyperproliferative effect on target tissues. Alternatively, a single vector encoding both genes may be used. A variety of proteins are encompassed within the invention, some of which are described below. Table 6 lists various genes that may be targeted for gene therapy of some form in combination with the present invention.

i. Inducers of Cellular Proliferation

The proteins that induce cellular proliferation further fall into various categories dependent on function. The commonality of all of these proteins is their ability to

regulate cellular proliferation. For example, a form of PDGF, the sis oncogene, is a secreted growth factor. Oncogenes rarely arise from genes encoding growth factors, and at the present, sis is the only known naturally-occurring oncogenic growth factor. In one embodiment of the present invention, it is contemplated that anti-sense mRNA directed to

5   a particular inducer of cellular proliferation is used to prevent expression of the inducer of cellular proliferation.

The proteins FMS, ErbA, ErbB and neu are growth factor receptors. Mutations to these receptors result in loss of regulatable function. For example, a point mutation

10   affecting the transmembrane domain of the Neu receptor protein results in the neu oncogene. The erbA oncogene is derived from the intracellular receptor for thyroid hormone. The modified oncogenic ErbA receptor is believed to compete with the endogenous thyroid hormone receptor, causing uncontrolled growth.

15   The largest class of oncogenes includes the signal transducing proteins (*e.g.,* Src, Abl and Ras). The protein Src is a cytoplasmic protein-tyrosine kinase, and its transformation from proto-oncogene to oncogene in some cases, results via mutations at tyrosine residue 527. In contrast, transformation of GTPase protein ras from proto-oncogene to oncogene, in one example, results from a valine to glycine mutation at amino

20   acid 12 in the sequence, reducing ras GTPase activity.

The proteins Jun, Fos and Myc are proteins that directly exert their effects on nuclear functions as transcription factors.

25   **ii.   Inhibitors of Cellular Proliferation**

The tumor suppressor oncogenes function to inhibit excessive cellular proliferation. The inactivation of these genes destroys their inhibitory activity, resulting in unregulated proliferation. The tumor suppressors p53, p16 and C-CAM are described below.

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High levels of mutant p53 have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is already documented to be the most frequently mutated gene in common human cancers.

5   It is mutated in over 50% of human NSCLC (Hollstein *et al.*, 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 393-amino acid phosphoprotein that can form complexes with host proteins such as large-T antigen and E1B. The protein is found in normal

10   tissues and cells, but at concentrations which are minute by comparison with transformed cells or tumor tissue

Wild-type p53 is recognized as an important growth regulator in many cell types. Missense mutations are common for the p53 gene and are essential for the transforming

15   ability of the oncogene. A single genetic change prompted by point mutations can create carcinogenic p53. Unlike other oncogenes, however, p53 point mutations are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the

20   germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Another inhibitor of cellular proliferation is p16. The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases, or CDK's. One CDK,

25   cyclin-dependent kinase 4 (CDK4), regulates progression through the G_1. The activity of this enzyme may be to phosphorylate Rb at late G_1. The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit, the p16^{INK4} has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus may regulate Rb phosphorylation (Serrano *et al.*, 1993; Serrano *et al.*, 1995). Since

30   the p16^{INK4} protein is a CDK4 inhibitor (Serrano, 1993), deletion of this gene may

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increase the activity of CDK4, resulting in hyperphosphorylation of the Rb protein. p16 also is known to regulate the function of CDK6.

p16^{INK4} belongs to a newly described class of CDK-inhibitory proteins that also includes p16^B, p19, p21^{WAF1}, and p27^{KIP1}. The p16^{INK4} gene maps to 9p21, a chromosome region frequently deleted in many tumor types. Homozygous deletions and mutations of the p16^{INK4} gene are frequent in human tumor cell lines. This evidence suggests that the p16^{INK4} gene is a tumor suppressor gene. This interpretation has been challenged, however, by the observation that the frequency of the p16^{INK4} gene alterations is much lower in primary uncultured tumors than in cultured cell lines (Caldas *et al.*, 1994; Cheng *et al.*, 1994; Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Kamb *et al.*, 1994; Mori *et al.*, 1994; Okamoto *et al.*, 1994; Nobori *et al.*, 1995; Orlow *et al.*, 1994; Arap *et al.*, 1995). Restoration of wild-type p16^{INK4} function by transfection with a plasmid expression vector reduced colony formation by some human cancer cell lines (Okamoto, 1994; Arap, 1995).

Other genes that may be employed according to the present invention include Rb, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, zac1, p73, VHL, MMAC1 / PTEN, DBCCR-1, FCC, rsk-3, p27, p27/p16 fusions, p21/p27 fusions, anti-thrombotic genes (*e.g.*, COX-1, TFPI), PGS, Dp, E2F, *ras, myc, neu, raf, erb, fms, trk, ret, gsp, hst, abl*, E1A, p300, genes involved in angiogenesis (*e.g.*, VEGF, FGF, thrombospondin, BAI-1, GDAIF, or their receptors) and MCC.

iii.   Regulators of Programmed Cell Death

Apoptosis, or programmed cell death, is an essential process for normal embryonic development, maintaining homeostasis in adult tissues, and suppressing carcinogenesis (Kerr *et al.*, 1972). The Bcl-2 family of proteins and ICE-like proteases have been demonstrated to be important regulators and effectors of apoptosis in other systems. The Bcl-2 protein, discovered in association with follicular lymphoma, plays a prominent role in controlling apoptosis and enhancing cell survival in response to diverse

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apoptotic stimuli (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Cleary *et al.*, 1986; Tsujimoto *et al.*, 1985; Tsujimoto and Croce, 1986). The evolutionarily conserved Bcl-2 protein now is recognized to be a member of a family of related proteins, which can be categorized as death agonists or death antagonists.

Subsequent to its discovery, it was shown that Bcl-2 acts to suppress cell death triggered by a variety of stimuli. Also, it now is apparent that there is a family of Bcl-2 cell death regulatory proteins which share in common structural and sequence homologies. These different family members have been shown to either possess similar functions to Bcl-2 (*e.g.*, Bcl_{XL}, Bcl_W, Bcl_S, Mcl-1, A1, Bfl-1) or counteract Bcl-2 function and promote cell death (*e.g.*, Bax, Bak, Bik, Bim, Bid, Bad, Harakiri).

e.   Surgery

Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present invention, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and miscopically controlled surgery (Mohs' surgery). It is further contemplated that the present invention may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

Upon excision of part of all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may

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be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

5   **f.   Other agents**

It is contemplated that other agents may be used in combination with the present invention to improve the therapeutic efficacy of treatment. These additional agents include immunomodulatory agents, agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell 10   adehesion, agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers, or other biological agents. Immunomodulatory agents include tumor necrosis factor; interferon alpha, beta, and gamma; IL-2 and other cytokines; F42K and other cytokine analogs; or MIP-1, MIP-1beta, MCP-1, RANTES, and other chemokines. It is further contemplated that the upregulation of cell surface receptors or their ligands such 15   as Fas / Fas ligand, DR4 or DR5 / TRAIL (Apo-2 ligand) would potentiate the apoptotic inducing abililties of the present invention by establishment of an autocrine or paracrine effect on hyperproliferative cells. Increases intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring hyperproliferative cell population. In other embodiments, cytostatic or 20   differentiation agents can be used in combination with the present invention to improve the anti-hyerproliferative efficacy of the treatments. Inhibitors of cell adehesion are contemplated to improve the efficacy of the present invention. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to 25   apoptosis, such as the antibody c225, could be used in combination with the present invention to improve the treatment efficacy.

Apo2 ligand (Apo2L, also called TRAIL) is a member of the tumor necrosis factor (TNF) cytokine family. TRAIL activates rapid apoptosis in many types of cancer 30   cells, yet is not toxic to normal cells. TRAIL mRNA occurs in a wide variety of tissues.

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Most normal cells appear to be resistant to TRAIL's cytotoxic action, suggesting the existence of mechanisms that can protect against apoptosis induction by TRAIL. The first receptor described for TRAIL, called death receptor 4 (DR4), contains a cytoplasmic "death domain"; DR4 transmits the apoptosis signal carried by TRAIL. Additional

5   receptors have been identified that bind to TRAIL. One receptor, called DR5, contains a cytoplasmic death domain and signals apoptosis much like DR4. The DR4 and DR5 mRNAs are expressed in many normal tissues and tumor cell lines. Recently, decoy receptors such as DcR1 and DcR2 have been identified that prevent TRAIL from inducing apoptosis through DR4 and DR5. These decoy receptors thus represent a novel

10   mechanism for regulating sensitivity to a pro-apoptotic cytokine directly at the cell's surface. The preferential expression of these inhibitory receptors in normal tissues suggests that TRAIL may be useful as an anticancer agent that induces apoptosis in cancer cells while sparing normal cells. (Marsters et al. 1999).

15   There have been many advances in the therapy of cancer following the introduction of cytotoxic chemotherapeutic drugs. However, one of the consequences of chemotherapy is the development/acquisition of drug-resistant phenotypes and the development of multiple drug resistance. The development of drug resistance remains a major obstacle in the treatment of such tumors and therefore, there is an obvious need for

20   alternative approaches such as gene therapy.

Studies from a number of investigators have demonstrated that tumor cells that are resistant to TRAIL can be sensitized by subtoxic concentrations of drugs/cytokines and the sensitized tumor cells are significantly killed by TRAIL. (Bonavida et al., 1999;

25   Bonavida et al., 2000; Gliniak et al., 1999; Keane et al., 1999). Ad-mda7 treatment of cancer cells results in the up-regulation of mRNA for TRAIL and TRAIL receptors. Therefore, administration of the combination of Ad-mda7 with recombinant TRAIL can be used as a treatment to provide enhanced anti-tumor activity. Furthermore, the combination of chemotherapeutics, such as CPT-11 or doxorubicin, with TRAIL also

30   lead to enhanced anti-tumor activity and an increase in apoptosis. The combination of

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Ad-mda7 with chemotherapeutics and radiation therapy, including DNA damaging agents, will also provide enhanced anti-tumor effects. Some of these effects may be mediated via up-regulation of TRAIL or cognate receptors, whereas others may not. For example, enhanced anti-tumor activity with the combinations of Ad-mda7 and tamoxifen

5   or doxorubicin (adriamycin) has been observed. Neither tamoxifen nor adriamycin are known to up-regulate TRAIL or cognate receptors.

Another form of therapy for use in conjunction with chemotherapy, radiation therapy or biological therapy includes hyperthermia, which is a procedure in which a

10   patient's tissue is exposed to high temperatures (up to 106°F). External or internal heating devices may be involved in the application of local, regional, or whole-body hyperthermia. Local hyperthermia involves the application of heat to a small area, such as a tumor. Heat may be generated externally with high-frequency waves targeting a tumor from a device outside the body. Internal heat may involve a sterile probe ,

15   including thin, heated wires or hollow tubes filled with warm water, implanted microwave antennae, or radiofrequency electrodes.

A patient's organ or a limb is heated for regional therapy, which is accomplished using devices that produce high energy, such as magnets. Alternatively, some of the

20   patient's blood may be removed and heated before being perfused into an area that will be internally heated. Whole-body heating may also be implemented in cases where cancer has spread throughout the body. Warm-water blankets, hot wax, inductive coils, and thermal chambers may be used for this purpose.

25   Hormonal therapy may also be used in conjunction with the present invention or in combination with any other cancer therapy previously described. The use of hormones may be employed in the treatment of certain cancers such as breast, prostate, ovarian, or cervical cancer to lower the level or block the effects of certain hormones such as testosterone or estrogen. This treatment is often used in combination with at least one

30   other cancer therapy as a treatment option or to reduce the risk of metastases.

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TABLE 6: Oncogenes

Gene	Source	Human Disease	Function
Growth Factors[1]			FGF family member
HST/KS	Transfection		
INT-2	MMTV promoter Insertion		FGF family member
INTI/WNTI	MMTV promoter Insertion		Factor-like
SIS	Simian sarcoma virus		PDGF B
Receptor Tyrosine Kinases[1,2]			
ERBB/HER	Avian erythroblastosis virus; ALV promoter insertion; amplified human tumors	Amplified, deleted squamous cell cancer; glioblastoma	EGF/TGF-α/ Amphiregulin/ Hetacellulin receptor
ERBB-2/NEU/HER-2	Transfected from rat Glioblastomas	Amplified breast, ovarian, gastric cancers	Regulated by NDF/ Heregulin and EGF-Related factors
FMS	SM feline sarcoma virus		CSF-1 receptor
KIT	HZ feline sarcoma virus		MGF/Steel receptor Hematopoieis
TRK	Transfection from human colon cancer		NGF (nerve growth Factor) receptor
MET	Transfection from human osteosarcoma		Scatter factor/HGF Receptor
RET	Translocations and point mutations	Sporadic thyroid cancer; familial medullary thyroid cancer; multiple endocrine neoplasias 2A and 2B	Orphan receptor Tyr Kinase
ROS	URII avian sarcoma Virus		Orphan receptor Tyr Kinase
PDGF receptor	Translocation	Chronic Myelomonocytic Leukemia	TEL(ETS-like transcription factor)/ PDGF receptor gene Fusion
TGF-β receptor		Colon carcinoma mismatch mutation target	
NONRECEPTOR TYROSINE KINASES[1]			
ABI.	Abelson Mul.V	Chronic myelogenous leukemia translocation with BCR	Interact with RB, RNA polymerase, CRK, CBL
FPS/FES	Avian Fujinami SV;GA FeSV		
LCK	Mul.V (murine leukemia virus) promoter insertion		Src family; T cell signaling; interacts CD4/CD8 T cells
SRC	Avian Rous sarcoma		Membrane-associated

Gene	Source	Human Disease	Function
	Virus		Tyr kinase with signaling function; activated by receptor kinases
YES	Avian Y73 virus		Src family; signaling

SER/THR PROTEIN KINASES[1]

Gene	Source	Human Disease	Function
AKT	AKT8 murine retrovirus		Regulated by PI(3)K?; regulate 70-kd S6 k?
MOS	Maloney murine SV		GVBD; cystostatic factor; MAP kinase kinase
PIM-1	Promoter insertion Mouse		
RAF/MIL	3611 murine SV; MH2 avian SV		Signaling in RAS Pathway

MISCELLANEOUS CELL SURFACE[1]

Gene	Source	Human Disease	Function
APC	Tumor suppressor	Colon cancer	Interacts with catenins
DCC	Tumor suppressor	Colon cancer	CAM domains
E-cadherin	Candidate tumor Suppressor	Breast cancer	Extracellular homotypic binding; intracellular interacts with catenins
PTC/NBCCS	Tumor suppressor and *Drosophilia* homology	Nevoid basal cell cancer syndrome (Gorline syndrome)	12 transmembrane domain; signals through Gli homogue CI to antagonize hedgehog pathway
TAN-1 Notch homologue	Translocation	T-ALI.	Signaling?

MISCELLANEOUS SIGNALING[1,3]

Gene	Source	Human Disease	Function
BCL-2	Translocation	B-cell lymphoma	Apoptosis
CBL	Mu Cas NS-1 V		Tyrosine-Phosphorylated RING finger interact Abl
CRK	CT1010 ASV		Adapted SH2/SH3 interact Abl
DPC4	Tumor suppressor	Pancreatic cancer	TGF-β-related signaling Pathway
MAS	Transfection and Tumorigenicity		Possible angiotensin Receptor
NCK			Adaptor SH2/SH3

GUANINE NUCLEOTIDE EXCHANGERS AND BINDING PROTEINS[3,4]

Gene	Source	Human Disease	Function
BCR		Translocated with ABL in CML	Exchanger; protein Kinase
DBL	Transfection		Exchanger
GSP			

Gene	Source	Human Disease	Function
NF-1	Hereditary tumor Suppressor	Tumor suppressor neurofibromatosis	RAS GAP
OST	Transfection		Exchanger
Harvey-Kirsten, N-RAS	HaRat SV; Ki RaSV; Balb-MoMuSV; Transfection	Point mutations in many human tumors	Signal cascade
VAV	Transfection		S112/S113; exchanger

NUCLEAR PROTEINS AND TRANSCRIPTION FACTORS[1,5-9]

Gene	Source	Human Disease	Function
BRCA1	Heritable suppressor	Mammary cancer/ovarian cancer	Localization unsettled
BRCA2	Heritable suppressor	Mammary cancer	Function unknown
ERBA	Avian erythroblastosis Virus		thyroid hormone receptor (transcription)
ETS	Avian E26 virus		DNA binding
EVII	MuLV promotor Insertion	AML	Transcription factor
FOS	FBI/FBR murine osteosarcoma viruses		1 transcription factor with c-JUN
GLI	Amplified glioma	Glioma	Zinc finger; cubitus interruptus homologue is in hedgehog signaling pathway; inhibitory link PTC and hedgehog
HMGI /LIM	Translocation t(3:12) t(12:15)	Lipoma	Gene fusions high mobility group HMGI-C (XT-hook) and transcription factor LIM or acidic domain
JUN	ASV-17		Transcription factor AP-1 with FOS
MLL/VHRX + ELI/MEN	Translocation/fusion ELL with MLL Trithorax-like gene	Acute myeloid leukemia	Gene fusion of DNA-binding and methyl transferase MLL with ELI RNA pol II elongation factor
MYB	Avian myeloblastosis Virus		DNA binding
MYC	Avian MC29; Translocation B-cell Lymphomas; promoter Insertion avian leukosis Virus	Burkitt's lymphoma	DNA binding with MAX partner; cyclin regulation; interact RB?; regulate apoptosis?
N-MYC	Amplified	Neuroblastoma	
L-MYC		Lung cancer	
REL	Avian Retriculoendotheliosis Virus		NF-κB family transcription factor
SKI	Avian SKV770		Transcription factor

Gene	Source	Human Disease	Function
	Retrovirus		
VHL	Heritable suppressor	Von Hippel-Landau syndrome	Negative regulator or elongin; transcriptional elongation complex
WT-1		Wilm's tumor	Transcription factor

CELL CYCLE/DNA DAMAGE RESPONSE[10-21]

Gene	Source	Human Disease	Function
ATM	Hereditary disorder	Ataxia-telangiectasia	Protein/lipid kinase homology; DNA damage response upstream in P53 pathway
BCL-2	Translocation	Follicular lymphoma	Apoptosis
FACC	Point mutation	Fanconi's anemia group C (predisposition leukemia	
FHIT	Fragile site 3p14.2	Lung carcinoma	Histidine triad-related diadenosine $5',3''''$-$P^1.p^4$ tetraphosphate asymmetric hydrolase
hMLI/MutL		HNPCC	Mismatch repair; MutL Homologue
hMSH2/MutS		HNPCC	Mismatch repair; MutS Homologue
hPMS1		HNPCC	Mismatch repair; MutL Homologue
hPMS2		HNPCC	Mismatch repair; MutL Homologue
INK4/MTS1	Adjacent INK-4B at 9p21; CDK complexes	Candidate MTS1 suppressor and MLM melanoma gene	p16 CDK inhibitor
INK4B/MTS2		Candidate suppressor	p15 CDK inhibitor
MDM-2	Amplified	Sarcoma	Negative regulator p53
p53	Association with SV40 T antigen	Mutated >50% human tumors, including hereditary Li-Fraumeni syndrome	Transcription factor; checkpoint control; apoptosis
PRAD1/BCL1	Translocation with Parathyroid hormone or IgG	Parathyroid adenoma; B-CLL	Cyclin D
RB	Hereditary Retinoblastoma; Association with many DNA virus tumor Antigens	Retinoblastoma; osteosarcoma; breast cancer; other sporadic cancers	Interact cyclin/cdk; regulate E2F transcription factor
XPA		xeroderma pigmentosum; skin cancer predisposition	Excision repair; photo-product recognition; zinc finger

B.   EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors

5   to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

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EXAMPLE 1: TRUNCATED MDA-7

1.   Materials and Methods

a.   Animals

3-6 wk-old female/male BALB/c nude mice were purchased from Harlan Inc.

15   (Indianapolis, IN).

b.   Virus

Control adenovirus (Ad-c) was prepared by deletion of E1 and partial E3 regions from adenovirus serotype 5. An adenovirus encoding human extracellular MDA-7

20   (Ad-mda7^{EC}) will be constructed by Introgen Therapeutics Inc., Houston, TX. Extracellular MDA-7 refers to the secreted portion of the MDA-7 protein.

c.   Cell preparation and infection with adenovirus

Where possible, cell lines are obtained from the American Type Culture

25   Collection (ATTC, Rockville, MD). The cells are grown in DMEM medium (GIBCO/BRL, Life Technologies, Grand island, NY) with 100 IU/ml penicillin, 0.1 mg/mL streptomycin and 10% fetal calf serum, HyClone, Logan, UT), according to ATCC's recommendation. The cells will be tested and verified to be free of mycoplasma and used in the log phase of growth. Cells are routinely harvested with 0.125% Trypsin –

30   1.3 mM EDTA (GIBCO).

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d. *In vitro* transfection

Cells are plated at a density of approximately 5×10^5 cells per 60 mm^2 in RPMI/10% FBS media and grown in 5% CO_2 at 37°C. Plating densities vary somewhat depending on cell growth rates etc. and are determined empirically.

e. Recombinant Adenovirus Production

Replication deficient human type 5 Adenovirus (Ad5) carrying the nucleic acid encoding extracellular human MDA-7 (or luciferase gene) linked to an internal CMV-IE promoter and followed by SV40 polyadenylation (pA) signal will be constructed. The adenovirus contruct encoding extracellular MDA-7 is designated Ad-mda7^{TF}, Ad-mda7^{EC}, and Ad-mda7^{TR}, which are used interchangeably to refer to an adenovirus construct encoding a portion of the full-length mda-7 gene sequence. Ad-mda7^{TF} will contain a nucleic acid sequence encoding a truncated transcript that encodes a truncated human MDA-7. An adenovirus (Ad5) construct encoding the full-length mda-7 gene sequence was made as described *infra* and used for some of these experiments.

The Ad-5 vectors harboring the gene cassettes will be co-transfected with plasmid pJM17 (Graham and Prevec 1992) in 293 cells to rescue recombinant viruses Ad-mda7^{TF}. Plaques will be picked, virus stocks will be grown and their genomes will be confirmed as correct by PCR/restriction analysis and sequencing. Viruses will be propagated in 293 cells and purified by chromatography. Control vectors including Ad-Luc (encoding the firefly luciferase gene), Ad-beta-gal (containing the bacterial beta-gal gene), Ad-GFP (containing the Green Fluorescent Protein) and Ad-CMVpA (containing the expression cassette, but no transgene) have been constructed and purified.

f. Transduction and Cell Proliferation studies

Cancer or normal cell lines used in this study will be infected with Ad-mda7^{TR} (with either AdCMVpA or AdLuc as controls) in increasing MOIs (multiplicities of infection) or viral particles/cell (0, 100, 250, 500, 1000, 2500, 5000, 10000 vp/cell

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increasing concentrations). Cells will be plated at 500-2000 cells/well in 96-well format for tritiated thymidine incorporation cell proliferation assay or plated at 10^5-10^6 cells/well in a 6 well plate for protein expression or apoptosis assays or plated at 10^4 cells/well for alamar-blue assay.

5

For infection Ad-mda7^{TR} or AdLuc (or AdCMVpA) will be used at increasing MOIs (based on viral particles/cell; MOI ranged from 0-10,000 viral particles/cell). For tritiated thymidine/apoptosis and protein expression and alamar assays, cells are analyzed 3 and 5 days post-infection

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g.   Tritiated-Thymidine Assay

Growth inhibition of cells after treatment will be measured by analysis of DNA-synthesis. Briefly, for the H^3-thymidine incorporation assay cells are plated at 200-5000 cell per well in a 96-well format and grown in DMEM/10% FBS in a 5% CO_2 incubator at 37°C overnight. The next day the media is aspirated and replaced with 50 μl DMEM/10% FBS containing the appropriate adenovirus at the appropriate MOI. Cells will be incubated with infecting media for 1 to 4 hrs and then diluted to 200 μl total volume and grown overnight. Media is replaced with DMEM/10% FBS/mCi H^3-thymidine and grown for 16 to 18 hrs. Stock solution of 100uCi/mL of H^3-thymidine (Amersham) is prepared by dilution into high glucose DMEM (GIBCO). H^3-Thymidine is added to each well at a final concentration of 1 μCi/mL. The reaction is stopped 15 hours later by removal of the supernatant from recipient cells. The cells are harvested by addition of 100x Trypsin/EDTA (GIBCO) to each well for 15 minutes at 37°C. Cells are collected on a filter in the 96-well format using a Packard Filtermate Cell Harvester following the manufacturer's protocol and washed in deionized water and methanol. The filter are dried and analyzed in Matrix 9600 (Packard) and cell proliferation using Viral Particles/cell against Tritiated Thymidine uptake counts are plotted.

h. Alamar Blue Assay

Growth inhibition of cells also will be measured by alamar blue assay. Briefly, cells are plated at 10^4cells/well density in a 96-well plate format. Four days after infection with different MOIs of Ad-mda7^{TR} or control vectors (as previously described), 20 µL of alamar blue dye is added to each well and the plate is incubated at 37°C for 6-8 hours. The plates are then read for optical density on the Dynatech MRX plate reader at wavelength of 595 nm. Revelation 3.2 software program is used to plot MOIs against optical density values at 595 nm.

i. TUNEL Assay

Cancer cells will be seeded in Lab-Tek chamber slides (Nunc) at density of 10^4 cells/chamber. Cells are transduced with desired concentration of Ad vectors. At different day points, post-infection, cells are analyzed according to manufacturer's instruction for apoptosis using the Chromogenic TUNEL-peroxidase assay ("In Situ Death Detection Kit, POD", Boehringer Mannheim).

j. Annexin V Assay

Cancer cells will also be analyzed for Apoptosis, post-Ad-mda7^{TR} treatment, by ApoAlert Annexin V-FITC kit (CLONTECH). After induction of apoptosis in cells, phosphatidylserine (PS), which is predominantly located on the inner leaflet of the plasma membrane, is rapidly translocated to the outer leaflet via a flippase mechanism. In the presence of Ca^{2+}, annexin V binds PS with high affinity and FITC conjugated to Annexin help to pinpoint apoptotic cells both via flourescent microscopy and FACs analysis.

k. DNA staining with Propidium Iodide (PI)

For determining cells at different stages of cell cycle, Ad-mda7^{TR}-infected cancer cells will be prepared as a single cell suspension of 1-2 x 10^6 cells/mL of PBS. After the cells are fixed with cold 70% ethanol for 2 hours, the cells are centrifuged, and the fixative decanted, and washed 2x with PBS and then stained with propidium iodide

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working solution, which includes PI at 50 μg/mL and RNAse at 20 μg/mL in PBS. Treated cells are then analyzed by FACS.

1.   Tumor Xenograft Models

5   Tumor cells are plated at a density of approximately 20-40% confluency in 150 mm^2 dishes in RPMI/10% FBS media supplemented with penicillin, streptomycin and fungizone, and grown in 5% CO_2 at 37°C until approximately 80% confluent. Cells are washed twice in PBS, trypsinized, and counted. Cells are diluted to a concentration of 5 x 10^6 cells/100 μl in PBS. BALB/c nude mice will be injected subcutaneously with 5 x

10   10^6 tumor cells in 100 μl of PBS.

2.   Adenovirus Encoding the Truncated Human MDA-7 Protein (Ad-mda7^{TF}) Produces a Secreted Protein

Crude cell fractionation studies will be done to demonstrate that MDA-7^{TF} is

15   secreted from cells transduced by Ad-mda7^{TF}. MDA-7^{TF} refers specifically to a truncated form of the MDA-7 protein; it includes the secreted form of MDA-7. Cells are plated as described and grown in DMEM/10% FBS in a 5% CO_2 incubator at 37°C overnight. The next day media is removed by aspiration and replace with fresh media containing Ad-mda7^{TF} or Ad-control. Media is aspirated after a 3 hr incubation with adenovirus and

20   replaced with fresh media. Cells are returned to the 5% CO incubator to be grown overnight at 37°C. Cells are harvested the next day and media is harvested by centrifugation. Western blot analysis to detect the MDA-7 protein is performed on cell extracts and total media. This protocol was used with Ad-mda7, and two immunoreactive bands were detected on the western blot using anti-MDA-7 antibody. The bands were

25   approximated to be 23kDa and 18kDa, which correspond with the unprocessed and processed forms of MDA-7 (full-length MDA-7 and the intra-cellularly cleaved form of MDA-7).

3. MDA-7 Is Localized to Vesicles and the Cell Surface by Confocal Microscopy

Expression of MDA-7 was also analyzed by FACS and Confocal microscopy. In brief, cancer cells (H460, DLD-1, H1299) were transduced with Ad-mda-7 at MOIs of 1000 and 5000 Vp/cell. 24 hours later the cells were washed with PBS and labeled with anti-mda-7 rabbit polyclonal antibody (1:5000 dilution of 1 mg/ml affinity purified antibody obtained from Corixa Inc.) for 1 hour at 4°C. The first treatment was followed by series of PBS washes and secondary anti-rabbit IgG-FITC (1:1000 dilution, Santa-Cruz Biotechnology), which was incubated with the cells for 1 hour on ice. The cells were washed and fixed with 4% HCHO-PBS and analyzed by FACS and Confocal Microscopy.

4. Elevated Expression of MDA-7^{TR} *in vitro* Induces Cancer Cell-Specific Growth Arrest and Apoptosis

Expression of MDA7^{TR} will inhibit growth and induce apoptosis in cancer cell lines but not in normal cell lines. Ad-mda7^{TR} will be transduced as previously described. Growth inhibition will be evaluated by ^{3}H-thymidine incorporation and alamar blue assays. Cancer cells treated with MDA7^{TF} will show a reduction in the rate of ^{3}H-thymidine incorporation as compared to normal cells transduced with mda7^{TF} and vector controls. Alamar blue assays will demonstrate a reduced optical density in MDA7^{TF}-expressing normal cells and transduced controls as compared to MDA7^{TF}-expressing cancer cells. These assays will be indicative of MDA7^{TF} expression specifically growth arresting cancer cells.

In addition, apoptosis will be evaluated by using TUNEL assay, surface expression of Annexin V, and cell cycle analysis using propidium iodide staining. The TUNEL assay evaluates cellular nuclease activity. Activated nuclease activity, indicative of cells undergoing apoptosis, will produce an intense color reaction in comparison with the background staining associated with non-apoptotic cells. Cancer cell lines expressing MDA-7^{TF} will demonstrate a significant increase in the number TUNEL positive cells as

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compared to mda-7^{TF}-expressing normal cell lines and control vector transduced cells. Another measure of apoptotic activity is the surface expression of annexin V as detected by surface staining using an annexin V anti-body. Cancer cell lines expressing mda7^{TF} will demonstrate a significant increase in the number of Annexin V positive cells as compared to mda-7^{TF}-expressing normal cell lines and control vector transduced cells, indicating an increased rate of apoptosis in the treated cancer cell lines as compared with normal cells and control treated cancer cells.

5. Intratumoral Administration of Ad-mda7^{TF} to H1299 Subcutaneous Tumors Inhibits Tumor Growth

For intratumoral delivery of Ad-mda7^{TF} 3-4 week old female BALB/c nude mice will be subcutaneously injected with 5x10^6 H1299 cells per animal. Animals will be treated five days post-H1299 injection by intratumoral injection of 100 μl of Ad-mda7^{TF}. Tumors are measured every other day. Treatment groups will be 1) no treatment 2) intratumoral injection every day for 6 days with Ad-mda7^{TF} 3) intratumoral injection every day for 6 days with Ad-control. Tumor size is measured every other day for 16 days. Ad-mda7^{TF} DNA will show a significant inhibition of tumor growth compared to no treatment and DNA alone.

EXAMPLE 2: MATERIALS AND METHODS

1. Cell Lines and Cell Culture

All the tumor cell lines utilized were obtained from American Type Culture Collection (ATCC, Rockville, MD). The cell lines evaluated were: breast (MCF-7, T47D, SKBr3, HBL-100, BT-20, MDA-MB-231, MDA-MB-468, MDA-MB-361), colorectal (DLD-1, SW-620, SW-480, HT-29, HCT-116, LS174T), lung (H1299, H460, A549, H322, H358) and osteosarcoma (SaosLM2) cancer lines. Normal cell lines were obtained from the Clonetics (San Diego) and included HUVEC (human umbelical vein endothelial cells), normal melanocytes, and HMEC (human mammary epithelial cells). MJ90 primary fibroblasts (obtained from Baylor College of Medicine) were also used.

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The cells were grown in DMEM medium (GIBCO/BRL, Life Technologies, Grand Island, NY) and fetal bovine serum (at 5-10% final concentration, according to suppliers recommendation). The cells were free of mycoplasma and were used in the log phase of growth. Cells were harvested with 0.125% Trypsin −1.3 mM EDTA (GIBCO).

5

2. Recombinant Adenovirus Production

The construction of Ad-mda7 was initiated by using a construct obtained from Dr. Paul Fisher (Columbia University, NY) that has the mda-7 cDNA inserted into a TA cloning vector (Invitrogen Inc., San Diego CA). The mda-7 cDNA, isolated as a Hind III-Not I

10 fragment, was cloned into a shuttle vector (pIN147, obtained from the laboratory of J.A. Roth, M.D.Anderson Cancer Center, Houston, TX) using the Hind III-Not I restriction sites. pIN147 is a shuttle plasmid that is based on a pBR322 backbone and contains the CMV promoter and SV40 poly A elements substituted into the E1 region of Ad 5. pIN147 contains bases #1-456 of the left end of Ad5, the CMV expression cassette and

15 then Ad5 bases #3333-5789. The pIN147-mda-7 cDNA expression construct was named pIN207. Ad-mda7 was constructed by co-transfecting 293 cells with pIN207 and pIN153 using the calcium phosphate kit and protocol provided by GibcoBRL (#123, pp44-46). pIN153 is identical to JM17 obtained from the laboratory of Graham, 1988. (A simple technique for the rescue of early region 1 mutations into infectious human adenovirus

20 type 5, McGrory *et al.*, 1988) and contains most of the Ad5 genome, but lacks the left end. After observing cytopathic effect (CPE), the resulting cell lysate that contained the vector, Ad-mda7, was subjected to two sequential rounds of plaque purification using the following protocol. Briefly, the vector-containing lysate was serially diluted in media (DMEM) in five-fold increments and 400 μl of each dilution was used to infect a

25 confluent layer of 293 cells plated on 6-well plates. The wells were overlayed with 1% agar in media and the resulting plaques were picked one week later. Each of four plaques was resuspended in 500 μl media, vortexed, centrifuged and the supernatant used to infect one well of confluent 293 cells plated on 6-well plates. The cells were allowed to undergo lysis due to Ad vector replication and DNA from each expanded primary plaque

30 was purified using the QIAmp DNA purification kit and protocol provided by Qiagen,

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Inc. The DNA was examined by PCR analysis, and one plaque was picked to undergo secondary plaque purification, which was performed as above. Plaques were isolated and used to infect 293 cells (as above) in one well of a 6-well plate; this lysate was then used to infect confluent 293 cells plated on a T-75 flask.

5

Finally, approximately 5×10^8 cells were infected from the resulting cell lysate Lysate from this sample was then transferred to manufacturing for large-scale amplification and purification. Ad-mda7 (lot #B2119901) and Ad-luc (lot #00697004) were used in the preclinical studies below. Early research studies used Ad-mda7 vector

10 obtained from Dr. Paul Fisher, Columbia University. The Fisher vector uses a similar first generation Ad vector with E1 and E3 deletions. This material was purified and amplified at Introgen Therapeutics Inc and shown to be bioequivalent to the Introgen Ad-mda7 used in all subsequent experiments.

15 Ad-Luc and Ad-CMVp(A) (Luciferase and empty vector, respectively), were used as control viruses (FIG. 1). For generation of Ad-vectors, Ad5 vectors harboring the gene cassettes were co-transfected with plasmid pJM17 (Graham and Prevec 1992) in 293 cells to rescue recombinant Ad-mda7, Ad-Luc and Ad-CMVp(A) viruses. Plaques were picked, virus stocks were grown, and their genomes were confirmed as correct by

20 PCR/Restriction analysis and DNA sequencing. Viruses were propagated in 293 cells and purified by HPLC.

3. MDA-7 Polyclonal Antibodies and Western Blot Analysis

Recombinant MDA-7 protein was produced in *E. coli* and was purified on a nickel

25 NTA agarose column. The material was bound to the nickel resin in a batch mode for 45 minutes and then poured into a column and the eluate was run through the column bed. The material was washed with 10 mM Tris pH 8.0 containing 0.5% chaps and finally eluted off of the column with 10 mM Tris pH 8.0 plus 400 mM imidazole. The eluted MDA-7 was dialyzed against 10 mM Tris pH 8.0. The final product was shown to be a

single band with a molecular weight of approx. 23 kDa. The amino terminal protein sequence was shown to be correct and purity was estimated to be greater than 90%.

This material was injected into rabbits using the following protocol: 400 µg MDA-7 protein with IFA and 100 µg of MDP was injected subcutaneously, 3 weeks later 200 ug MDA-7 protein with IFA was injected and 3 weeks after that another 100 µg of MDA-7 protein was injected intravenously. The titer of antiserum was shown to be greater than 1/100,000 based on an ELISA assay.

The MDA-7 protein was coupled via sulfhydryl linkage to a solid support resin. The resin and bound protein was thoroughly washed. This washed material was used to make an MDA-7 column for antibody purification. The rabbit polyclonal sera was diluted 1:1 with 20 mM Tris buffer pH 8.0 and filtered through a 0.2-micron filter before being pumped onto the MDA-7 column. The column was then washed with the same 20 mM Tris buffer pH 8.0 until the absorbance returned to baseline. The antibody was eluted off the column with 0.1 M acetic acid. The eluent containing the antibody was immediately adjusted back to pH 8.0. This affinity-purified antibody was then dialyzed against 10 mM Tris pH 8.0 and concentrated.

Recombinant MDA-7 protein was expressed in *E. coli* and purified using a nickel NTA agarose column. This recombinant MDA-7 protein was used to generate rabbit polyclonal antibodies, which were purified by affinity chromatography. This antibody was used in Western blot analysis at concentrations ranging from 1:1000-1:10,000 dilution (from stock of 1 mg/mL). Cell lysates (10^5-10^6 cells were suspended in 500 µL of Laemmli buffer with 5% 2-mercaptoethanol[2ME]) or supernatants (1:1 mixing with Laemmli buffer +2ME), were obtained after cancer cells were treated with Ad-mda7 for desired length/s of time, followed by SDS polyacrylamide gel electrophoresis and western blot analysis using the Super-Signal substrate for horseradish peroxidase (Pierce Inc.). Other monoclonal antibodies used in the study specifically recognized Bax (Santa Cruz Biotechnology) and β-actin (Sigma).

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4. Transduction and Cell Proliferation Studies

Cancer or normal cell lines used in this study were infected with Ad-mda7 (using Ad-CMVp(A) or Ad-Luc as controls) with increasing MOIs (0 100, 250, 500, 1000, 2500, 5000 and 10,000 viral particles (vp) /cell). Cells were either plated at 500-2000 cells/well in 96-well format for ^{3}H-thymidine incorporation-assay, or plated at 10^5-10^6 cells/well in a 6-well plate format for protein expression or apoptosis assays, and plated at 10^4 cells/well (96-well format) for Alamar-blue assay. For infection, Ad-mda7 or Ad-Luc (or Ad-CMVp(A) were used at increasing MOIs (based on viral particles/cell; MOI ranged from 0-10,000 vp/cell). For ^{3}H-thymidine-incorporation, apoptosis, protein expression and Alamar assays, cells were analyzed 3 and 5 days post-infection

5. ^{3}H thymidine Assay

Growth inhibition of cells after treatment was primarily measured by analysis of incorporation of ^{3}H-thymidine into replicating DNA. Briefly, a stock solution of 100 µCi/mL of ^{3}H-thymidine (Amersham) was prepared by dilution into high glucose DMEM (GIBCO). ^{3}H-thymidine was added to each well at a final concentration of 1 µCi/mL. The reaction was stopped 15 hours later by removal of the supernatant from recipient cells. The cells were harvested by addition of 100x trypsin/EDTA (GIBCO) to each well for 15 minutes at 37°C. Cells were collected on a filter using a Packard Filtermate Cell Harvester following the manufacturer's protocol and washed in deionized water and methanol. The filters were dried and analyzed using a Matrix 9600 (Packard).

6. Alamar Blue Assay

Growth inhibition of cells was also measured using the Alamar Blue Assay. Cells were plated at 10^4 cells/well in a 96 well plate format. Four days after infection with different MOIs of Ad-mda7 or control vectors (as mentioned earlier), 20 µL of alamar blue dye was added to each well and the plate was incubated at 37°C for 6-8 hours.

The plates were then processed for optical density absorbance analysis using the Dynatech MRX plate reader at dual wavelengths of 575 and 600nm. Revelation 3.2 software program was used to analyze data.

7.   **TUNEL Assay**

Cancer cells were seeded in Lab-Tek chamber slides (Nunc) at density of 10^3-10^4 cells/chamber. Cells were transduced with desired concentration of Ad-vectors. At different time-points after infection, cells were analyzed according to manufacturer's instruction for apoptosis-induction using the Chromogenic TUNEL-POD (Boehringer Mannheim) assay. Cells were analyzed 2-5 days post-infection. The kit utilizes a deoxythymidine transferase (TdT) enzyme to incorporate fluorescein bound-deoxythymidine molecules to fragmented DNA with free hydroxyl groups. After washing with PBS, horseradish peroxidase (POD)-tagged-anti-fluorescein antibody is used as the secondary agent, and samples are exposed to DAB to identify TUNEL positive cells (dark brown staining).

8.   **Annexin V Assay**

Cancer cells were also analyzed for apoptosis, using the ApoAlert Annexin V-FITC kit (CLONTECH). Ad-vector-transduced cells (10^5-10^6 cells total) were washed extensively in PBS and then incubated with Annexin V-FITC reagent for 30 minutes on ice. Cells are then washed and processed for FACS analysis and fluorescent microscopy.

9.   **Hoechst Protein Staining**

Hoechst dye (33258) was a product of ICN Biomedicals (Ohio,USA). Briefly, the cells in chamber slides were fixed (methanol:acetic acid = 3:1) for 5 min, and then fixed again with the same fixative for 10 min. They were air-dried for 30 min, and then placed in 1.0 ml staining solution (0.05 mg/ml Hoechst 33258 in 1 x PBS buffer) for 30 min, followed by washing three times (1 min each) with distilled water. After the washes, the slides were air-dried, and photos were taken under a fluorescence microscope or analysed by confocal microscopy.

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10.   DNA Staining with Propidium Iodide (PI)

Cell-cycle staging was done by the evaluation of DNA content by PI staining. For identifying cells at different stages of cell cycle, vector infected cancer cells were prepared as a single cell suspension of 1-2 x 10^6 cells/mL of PBS. After the cells were fixed with cold 70% ethanol for 2 hours, the cells were centrifuged, the fixative decanted, and cells washed 2x with PBS and then stained with propidium iodide (PI) at final concentration of 50 μg/mL with RNAse at 20 μg/mL in PBS. Treated cells were then analyzed by FACS analysis.

J.   Surface Expression Studies

Cancer cell lines were treated with increasing MOIs (multiplicities of infection) of Ad-mda7 or Ad-luc as control. Briefly, 1 x 10^6 cells (in 6-well plate) were infected with Ad vectors and 48 h later the cells were dislodged with 500 μL of Versene. The cells were washed 3x with PBS (3 mL per wash) and treated with 500 μL of 1:2000 diluted rabbit anti-mda7 affinity purified antibody (stock conc. 1mg/mL) at 4°C for 2h. After this treatment, the cells were washed with PBS (3x) and treated with 1:1000 diluted goat anti-rabbit IgG-FITC for 2h at 4°C. Cells were washed, fixed using 4% formaldehyde in PBS and analyzed by FACS analysis.

The Fluorescent Microscopy and Imaging Core Facility at UT Health Science Center was used for the confocal work described in this report. The following center systems were used: Molecular Dynamics 2001 CSLM (Confocal Scanning Laser Microscope), Applied Precision Deltavision Deconvolution Microscope, Nikon 8000 RSCM (Real time scanning Confocal microscope) and Wallac/Olympus Concord system (real time fluorescence imaging system).

For MDA-7 protein staining, cells were infected with Ad-mda7 (or Ad-luc) at an MOI of 1000 vp/cell. After 48 hours, the cells were washed 3x with PBS (3 mL per wash) and treated with 500 μL of 1:2000 diluted rabbit anti-Mda-7 affinity purified

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antibody (stock conc. 1mg/mL) at 4°C for 2h. After the treatment, the cells were washed with PBS (3x) and treated with 1:1000 diluted goat anti-rabbit IgG- rhodamine. The cells were also stained with Annexin V and Hoechst stain. Cells were fixed post-staining using 4% formaldehyde in PBS.

For intracellular [Ca^{2+}] characterization, Ad-mda7 transduced cells were loaded for 15min in the dark at 37°C with the calcium probe FLUO3 at final concentration of 2 μM (Minta *et al.* 1989, Molecular Probes, Eugene, OR), then visualized with a Molecular Dynamics scanning laser confocal microscope at wavelength of 488 nm. Areas and volumes of the cells were determined using Image Space software (Molecular Dynamics, Sunnyvale, CA), following optical stack sectioning of the cells. Fast scan image captures were made to visualize calcium waves passing through the Ad-mda7 transduced cancer cells, and these images were then compiled and sequenced.

Mitochondrial content was determined using similar protocols as above (Ca^{2+} experiments), except the probe used was MitoTrack (Molecular Probes). Loading parameters and probe concentration were the same as FLUO 3, with the scans performed at a wavelength setting of 595 nm (Marin et al. 1996).

K.   Glycosylation Analyses

Supernatant was treated with the following three enzymes either individually or in different combinations. The enzymes used were sialidase (neuraminidase), endoglycosidase-H and endoglycosidase-F (all obtained from Sigma). The buffer conditions were 100 mM Tris, pH 8.2. For every microgram of total supernatant protein used, approximately 0.1 units of enzyme/s were used. The enzymes used were Sialidase (Neuraminidase), Endoglycosidase-H and N-glycosidase-F (all obtained from Sigma). The buffer conditions were 100 mM Tris, pH 8.2. The reaction was carried out at 37°C for one hour and then the samples were run on SDS-PAGE and analyzed by Western blot using the specific anti-MDA7 rabbit polyclonal antibody.

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EXAMPLE 3: AD-MDA7 KILLS CANCER CELLS AND INDUCES APOPTOSIS

1.   Breast Cancer Cells

A series of breast cancer cell lines (T47D, MCF-7, BT-20, MDA-MB-361, SKBr3, MDA-MB-231, MDA-MB-468) were transduced with Ad-mda7 (or Ad-CMVp(A) or Ad-luc as control vectors). The cell lines were strongly growth-inhibited by Ad-mda7 transduction. The two cell lines that demonstrated the highest sensitivity to Admda7 were T47D (p53 mutated) and MCF-7 (p53 wild-type) (FIG. 2A and 2B), as determined by ^{3}H-thymidine incorporation assay. Cancer cells were analyzed 3-6 days after Ad-mda7 transduction. See TABLE 7 below.

TABLE 7: Summary of breast cancer lines used for Ad-mda7 studies.

Cell Line	Tumor type	p53 status	Source
Breast Cancer			
(1) T47D	ductal carcinoma	L194F	ATCC
(2) MCF-7	carcinoma	wt	ATCC
(3) MDA-MB-361	adenocarcinoma	wt	ATCC
(4) MDA-MB-231	adenocarcinoma	R280K	ATCC
(5) MDA-MB-468	adenocarcinoma	R273H	ATCC
(6) SKBr-3	adenocarcinoma	Mut	ATCC
(7) BT-20	carcinoma	Mut	ATCC
Normal			
(1) MJ90	fibroblast	wt	Smith lab
(2) HUVECs	endothelium	wt	Clonetics
(3) HMECs	mamm. epithelium	wt	Clonetics

FIG. 7 illustrates the high levels of apoptosis (as measured by Annexin V staining) induced in breast cancer cell lines by Ad-mda-7. Annexin V staining identifies cells in early and mid-stages of apoptosis, whereas the TUNEL assay detects DNA cleavage products, one of the final stages of apoptosis. TUNEL assays performed on

MCF-7 cells infected with Ad-mda7 confirmed that these cells are killed via apoptotic pathways. Ad-CMVp(A) or Ad-luc control vectors were ineffective at inducing apoptosis.

5   The two cell lines that demonstrated the highest sensitivity to Ad-mda7 were T47D (p53 mutant) and MCF-7 (p53 wild-type) (FIG. 2A and 2B). The Ad-mda7 concentration needed to inhibit growth by 50% (IC_{50}) of the T47D or MCF-7 cells averaged 500 and 1500 vp/cell, respectively (TABLE 8). Also included in FIG. 3 (Panels A and B) are representative experiments using MDA-MB-361 and BT-20 cells. These

10   two cell lines also showed marked sensitivity to Ad-mda7 infection. Table 8 summarizes the responsiveness of breast cancer cells to Ad-mda7 infection (as determined by a comparison of IC_{50} values for Ad-mda7 and control Ad vector). Also included in Table 8 are the IC_{50} values in normal cell lines.

Table 8: Summary of IC_{50} values of Ad-mda7 in Breast Cancer and Normal lines

Cell Line	Tumor type	IC$_{50}$ range	
		Admda-7	Control*
Breast Cancer			
(1) T47D	ductal carcinoma	150-500	>10,000
(2) MCF-7	carcinoma	1200-4000	>10,000
(3) MDA-MB-361	adenocarcinoma	~1500	>10,000
(4) MDA-MB-231	adenocarcinoma	~3000	>10,000
(5) MDA-MB-468	adenocarcinoma	>10,000	>10,000
(6) SKBr-3	adenocarcinoma	~5000	>10,000
(7) BT-20	carcinoma	~2500	>10,000
Normal			
(8) MJ90	fibroblasts	>10,000	>10,000
(9) HUVECs	endothelium	>10,000	>10,000
(10) HMECs	mamm. epithelium	>10,000	>10,000

* The control vectors used in these experiments were either Ad-CMVp(A) or Ad-luc.

2. AD-MDA7 KILLS LUNG CANCER CELLS AND INDUCES APOPTOSIS

Six lung cancer lines (H1299, H460, A549, H322, H358 and SaosLM2) were infected with Ad-mda7. All of these demonstrated effective killing by Ad-mda7 transduction. The H1299, and H322 cell lines were the most sensitive to Ad-mda7 killing (see FIG. 4A and B). The IC_{50} in these lines ranged from 600 vp/cell to 2000 vp/cell as determined by ^{3}H-thymidine incorporation assay.

3. AD-MDA7 KILLS COLORECTAL CANCER CELLS AND INDUCES APOPTOSIS

Six colorectal cancer lines (DLD-1, SW-620, SW-480, HT-29, HCT-116, LS174T) were infected with Ad-mda7. All of these cell lines were effectively growth inhibited by Ad-mda7 transduction, with SW620, DLD-1 and SW-480 being the most sensitive. SW620 cells treated with Ad-mda7 at varying MOIs is shown in FIG. 5A,

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while DLD-1 cells are shown in FIG. 5B. Cell proliferation, as determined by ^{3}H-thymidine incorporation assay, demonstrated an IC_{50} that averaged 1000 vp/cell in the more sensitive cell lines to 2000 vp/cell in the other less-sensitive cell lines. The DLD-1 cell line was infected with Ad-mda7 at 1000 and 5000 vp/cell, using uninfected cells and Ad-Luc as controls. Forty-eight hours later the transduced cells were analyzed for apoptosis using Annexin V staining in conjunction with FACS analysis. Neither the uninfected or AdLuc-infected (5000 vp/cell) cells showed signs of apoptosis, whereas Ad-mda7 infected cells exhibited approximately 26% apoptotic cells at 1000 vp/cell and 58% apoptotic cells at 5000 vp/cell (FIG. 8).

4. AD-MDA7 INFECTION IN NORMAL CELLS

Three normal human cell lines (MJ90 fibroblasts, HUVEC endothelial cells and human mammary epithelial cells) showed no growth inhibition when infected with Ad-mda7. The primary fibroblast cell line MJ90 showed overlapping growth curves when treated with Ad-mda7 or Ad-luc control vector (FIG. 6A). HUVEC and human mammary epithelium cells showed similar results (FIG. 6B).

5. PROTEIN ANALYSES

Cell lysates obtained from Ad-mda7 transduced cancer cell lines were size fractionated by SDS-PAGE followed by western-blot analysis using a rabbit anti-MDA7 antibody. The migration of the MDA-7 protein was consistent with an approximate size of 23 kD, however, an additional band at 17 kD was also observed. A Western blot analysis of H1299 (lung cancer) and DLD-1 (colorectal cancer) cell lines was performed after Ad-mda7 and Ad-luc infection. Two bands at approximately 23 and 17 kD were observed. Similar molecular weight size bands were also seen in breast cancer lines infected with Ad-mda7. During the first 48 hours post-infection the 17 kD band was the major species observed in DLD-1 cells. At 72 and 96 hours post infection, the intensity of the 23 kD band decreased with time and other smaller degradation products were seen. In H1299 cells, both bands had similar intensities. The blots were also probed for β-

actin, and at 72 and 96 hours post-infection, actin was substantially degraded (data not shown), consistent with the rapid apoptotic death of cells.

As seen in these protein expression studies, lysates from Ad-mda7 infected cells show a 23kD/17 kDa doublet, suggesting that MDA-7 is processed intracellularly. Previous studies by Su *et al.*, 1998, indicated that in human melanoma cells induced with interferon β and mezerin, the 23 D MDA-7 protein translocated from the cytosol to the nucleus. On the basis of primary protein sequence analysis, MDA-7 does not possess any consensus nuclear localization motifs, which may suggest MDA-7 protein associating with a cytoplasmic chaperone (such as HMC) (Jiang *et al.*,1995, 1996). It was proposed that this association may facilitate the translocation of mda-7 into the nucleus.

6.   APOPTOSIS STUDIES

Annexin V staining identifies cells in early and mid-stages of apoptosis, whereas the TUNEL assay detects DNA cleavage products, one of the final stages of apoptosis. FIG. 7 illustrates the high levels of apoptosis (as measured by Annexin V staining) induced in breast cancer cell lines by Ad-mda7. TUNEL assays were performed on MCF-7 cells infected with Ad-mda7, thus confirming that these cells are killed via apoptotic pathways. Ad-CMVp(A) or Ad-luc control vectors were ineffective at inducing apoptosis.

Further examples of Ad-mda7-induced apoptosis are shown in Figures 10 and 11. The DLD-1 cell line was infected with Ad-mda7 at 1000 and 5000 vp/cell, using uninfected cells and Ad-Luc as controls. Forty-eight hours later the transduced cells were analyzed for apoptosis using Annexin V staining in conjunction with FACS analysis (FIG. 8). Neither the uninfected or Ad-Luc infected (5000 vp/cell) cells showed signs of apoptosis, whereas Ad-mda7 infected cells exhibited approximately 26% apoptotic cells at 1000 vp/cell and 58% apoptotic cells at 5000 vp/cell. Ad-mda7 caused rapid induction of apoptosis (FIG. 9). Two cell lines representing NSCLC and colorectal cancer are shown. Substantial levels of apoptosis were evident as soon as 12 hours post-infection

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with Ad-mda7, and increased over the next few days. The demonstration of apoptosis as soon as 12 hr post-infection is notable as immunoreactive MDA-7 protein is just detectable at 12 hr and, generally, does not peak until 24-48 hr post-infection. Ad-p53 can also cause rapid induction of apoptosis, however, other tumor suppressors, such as p16 or PTEN tend to cause apoptosis only after 2-3 days post infection with the Ad expression vector.

7.   Ad-mda7 Increases Bax Protein Levels in Lung, Breast and Colorectal Cancer Lines

Regulation of programmed cell death relies on the interaction between signaling pathways that either promote or inhibit apoptosis (Reed 1997; White 1996). The bcl-2 family members (bcl-2, bcl-w, bax, bad, bak, bcl-xs) play an important role in apoptotic signaling (Sedlak *et al.*1995; Reed *et al.*1996).   Using Western blot analysis in conjunction with an anti-bax antibody it was determined that Ad-mda7 infection upregulated the BAX protein in T47D, DLD-1, A549 and H460 cells. Western blot analysis of lysates prepared 24 hours after infection with 30 to 150 pfu/cell of Ad-mda7 demonstrated increased expression of BAX in all cell lines tested. For example, upregulation of BAX in Ad-mda7 infected T47D cancer cell line was observed by Western blot analysis. Cells were infected with Ad-mda7 and analyzed for MDA-7 and BAX protein expression. Ad-mda7 increased BAX expression in T47D, as was observed with the other cell lines.

8.   Endogenous Expression of Mda-7 in Cancer and Normal Cells

Of the more than 50 tumor cell lines evaluated for endogenous Mda-7 protein expression, only two, DLD-1 (colorectal) and LnCap (prostate) were positive. Studies are underway to look at mda-7 mRNA in the various cancer lines. Table 9 is a list of some of the cancer lines used in the Ad-mda7 studies and their endogenous MDA-7 status. There was no correlation between the anti-tumor activity of Ad-mda7 and MDA-7 endogenous expression based on Western blot analysis (TABLE 9).

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TABLE 9

Cell Type	Endogenous MDA-7 protein	Ad-mda7 killing
(A) Normal lines		
(1) MJ90	--	--
(2) HUVEC	--	--
(3) HMEC ·	--	--
(B) Breast cancer lines		
(1) T47D	--	++++
(2) MCF-7	--	+++
(3) MDA-MB231	--	++
(4) MDA-MB468	--	++
(C) Lung cancer lines		
(1) H1299	--	++++
(2) A549	--	++
(3) H460	--	++
(D) Colorectal cancer lines		
(1) DLD-1	++	+++
(2) SW620	--	+++
(3) HCT116	--	++
(4) HT29	--	++
(E) Prostate cancer lines		
(1) LnCap	++	+++
(2) Du145	--	++

Note: -- denotes undetectable endogenous protein/no response to Ad-mda7 infection; ++ denotes presence of endogenous mda7 protein or effective responsiveness to Ad-mda7.

9.   AD-MDA7 FUNCTIONS INDEPENDENTLY OF ENDOGENOUS P53, RB, RAS, AND P16 STATUS

Table 10 presents the status of different tumor suppressor/oncogene/cell cycle regulating genes and their response to Ad-mda7 infection in different cell lines used in this study. The growth-inhibitory action of MDA-7 was observed in a wide variety of cancer cell lines, independent of their p53, RB, p16, and Ras status. Although, Bax expression is positively regulated by wild-type p53 (Han et al., 1996), the ability of MDA-7 to induce BAX appears to be independent of p53 since BAX up-regulation is observed in p53-mutant (DLD-1, T47D) and p53-wild-type (H460). It is interesting to

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note that MDA-7 was able to effectively induce apoptosis in the MCF-7 breast cancer cells that are devoid of caspase 3, one of the several caspases involved in the downstream apoptotic events.

TABLE 10

Cell Type	Ad-mda7 effect	p53	RB	ras	p16
(A)Normal lines					
(1) Melanocytes	ND	wt	wt	wt	wt
(2) MJ90 fibroblasts	--	wt	wt	wt	wt
(3) HUVEC	--	wt	wt	wt	wt
(4) HMEC	--	wt	wt	wt	wt
(B) Cancer Lines					
(1) T47D	++++	mut	--	wt	--
(2) MCF-7	+++	wt	--	wt	--
(3) H1299	++++	null	wt	mut	--
(4) Saos-LM2	++	del	--	wt	del
(5) A549	++	wt	--	mut	--
(6) H460	++	wt	wt	mut	del
(7) SW620	+++	mut	--	mut	--
(8) HCT116	++	wt	--	mut	--

Note: ND, Not determined; mut, mutation; del, deletion; wt, wild-type

EXAMPLE 4: MDA-7 CELLULAR LOCALIZATION STUDIES

1. SURFACE EXPRESSION STUDIES

The H460 NSCLC cell line was treated with increasing MOIs of Ad-mda7 or Ad-luc as control, and 48 h later, the cells were stained with the polyclonal anti-MDA-7 antibody and analyzed by FACS analysis (FIG. 10). A high level of staining was observed in the Ad-mda7 treated cells only. The staining was dose-dependent and

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approximately 50% of cells were MDA-7 positive at 1000 vp/ cell. This result indicated that Ad-mda7 treatment of H460 cells resulted in high levels of protein production (verified by Western blot analysis) and that the protein appeared to on the cell surface.

5   **B.   Confocal Microscopy Studies**

To confirm and extend the results shown in FIG. 10, confocal microscopic analyses were performed on various cell lines (H460, H1299, T47D and DLD-1 cells) to determine sub-cellular distribution MDA-7 protein after Ad-mda7 treatment. Background staining in untreated or Ad-luc-treated cells was low and diffuse. The

10   background is believed to be due to the anti-MDA-7 reagent being a polyclonal antiserum. However, highly specific staining was observed when cells were treated with Ad-mda7. At low MOIs, distinct membrane staining was observed with punctate staining in the cytoplasm. At higher MOIs, the punctate staining and membrane staining were reproduced and more intense. The pattern of staining was suggestive of a secreted

15   protein, with the punctate staining representing protein trafficking and release at the plasma membrane. Similar observations were observed in the other cell lines

In additional confocal microscopy experiments, cancer cell lines were treated with Ad-mda7 and analyzed for apoptosis (Annexin V staining), DNA content (Hoechst),

20   Ca2+ influx/eflux (Fluo 3, Molecular Probes) and mitochondrial integrity (MitoTrack, Molecular Probes). The protocols used were those established in the Confocal Microscope Facility, UTHSC, Houston, TX.

Confocal microscopic studies of H460 and MCF-7 cells were done. They show a

25   composite of individual microscopic fields: (1) denotes surface expression of MDA-7 (red surface and punctate staining), (2) showing apoptosing cells (polarized green staining), (3) Hoechst staining to identify nuclei (blue) and (4) composite of (1) (2) and (3).

Calcium and mitochondrial staining was done in Ad-mda7- or Ad-luc control-transduced cells. Cells were plated on laminin-coated cover-slips and treated with FLUO-3 (for Ca^{2+}) or with Mitotracker (for mitochondria). The control Ad-luc treated cells show a well distributed intracellular calcium content (green fluorescence) and

5   displayed good mitochondrial integrity (red staining). However, on Ad-mda7 treatment, intracellular Ca^{2+} levels are disrupted and the mitochondrial integrity is disrupted.

EXAMPLE 5: SECRETED MDA-7 PROTEIN

10   ### 1.   Secretion of MDA-7

H1299 cells were infected with Ad-mda7 (MOI of 1000 vp/cell) for 6 hours, washed with fresh media and incubated at 37°C in fresh DMEM media. Twenty-four hours later, the cell lysate and the growth media were analyzed for MDA-7 protein expression using Western blot. Ad-mda7 transduced cells showed a specific 40 kD

15   protein produced in growth media, which was absent in untransduced or Ad-luc transduced cells that only showed 19 kD and 23 kD bands. A dose-dependent increase in the intra-cellular MDA-7 and the extra-cellular MDA-7 protein was observed. As a control, the blot in Panel B was probed with an anti-actin antibody. As predicted, the cell lysates showed an actin signal at approx. 40 kD, whereas the cell supernatants did not

20   show any actin signal. This suggests that the MDA-7 protein signal observed in the supernatants is due to active release/ secretion of MDA-7 and is not due to release from dying cells.

2.   Glycosylation of Secreted MDA-7 Protein

25   The supernatant from Ad-mda7 transduced H1299 cells was a good source of obtaining the secreted MDA-7 protein. The supernatant was further evaluated for protein glycosylation. Supernantant was treated with the following three enzymes either individually or in different combinations. The enzymes used were sialidase (neuraminidase), endoglycosidase-H and endoglycosidase-F (all obtained from Sigma).

30   The samples were analyzed by Western blot using the specific anti-MDA-7 rabbit polyclonal antibody.

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Endoglycosidase treatment suggests that soluble MDA-7 protein is glycosylated. Using various glycosidases, especially Endo F, a lower molecular weight band is also observed (which is approximately the same size as the MDA-7 protein band observed in cell lysate.

3.   Inhibition of Glycosylation and Secretion of MDA-7 Protein

Two antibiotics, Tunicamycin and Brefeldin A, have been used to provide a more detailed characterization of the secretion of soluble MDA-7. N-linked glycosylation plays an important role in a protein's ultimate processing, whether it is sorted to a lysosomal pathway, or translocated to the cell surface or secreted. Using Tunicamycin, the N-linked glycosylation process in the golgi apparatus can be inhibited, thus inhibiting protein secretion or other sugar-dependent sorting processes. Brefeldin A is a fungal metabolite (macrocyclic lactone) which exhibits a wide variety of antibiotic activities. Brefeldin A reversibly inhibits the intracellular translocation of proteins (during transport of protein to the cell surface for secretion or expression. Both Tunicamycin and Brefeldin A effectively inhibit the secretion of soluble MDA-7 protein. Therefore, intracellular processing and glycosylation appear to be required for MDA-7 secretion.

4.   Secreted MDA-7 Protein Induces Killing in Cancer Cells

The secreted form of MDA-7 (sMDA-7) was produced using various cell lines and evaluated for anti-tumor activity. A representative experiment is shown in FIG. 11. Soluble MDA-7 was analyzed for its anti-proliferative effects on H1299 cells. Briefly, H1299 cells were plated at cell density of 10^3 cells/chamber in Nunc chamber slides. 24 hours later, the cells were challenged with supernatants obtained from H1299 cells transduced with either Ad-mda-7 or Ad-luc (at 1000 vp/cell infection). Ad-mda7 and Ad-luc viruses were also used as additional controls. The soluble protein supernatants (500 uL total volume, different dilutions) were applied to naïve H1299 cells and 24 hours later an additional 0.5mL of 10% FBS in DMEM was added. After 24 and 48 hours, the cells were microscopically examined for viability using the trypan blue exclusion staining. The

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soluble MDA-7 protein showed H1299 killing after 48 hours; however, Ad-luc supernatants had little effect (FIG. 11A).

Various dilutions of soluble MDA-7 supernatant were also analyzed for H1299 killing using the Trypan blue exclusion assay. A concentration-dependent bystander killing effect of soluble MDA-7 was observed (FIG. 11B).

EXAMPLE 6: COMBINATION STUDIES OF AD-MDA7 IN BREAST CANCER LINES

1. Combination with Tamoxifen

Ad-mda7 has been combined with tamoxifen and evaluated for anti-tumor effects in breast cancer cell lines (FIG. 12). The graphs demonstrate that combining these two agents provides superior anti-tumor activity compared to either agent alone. The effect of tamoxifen on T47D cells is shown (FIG. 12A) and on MCF-7 cells (FIG. 12B). Cells were plated and four days after treatment, a tritiated thymidine assay was performed to measure DNA replication. Cells were treated with 0/0 (no drug and no vector) or varying doses of tamoxifen or vectors (Ad-luc or Ad-mda7). In T47D cells, tamoxifen or Ad-mda7 had minimal effect on DNA replication. However, when the tamoxifen and Ad-mda7 were combined, a supra-additive effect was observed. In MCF-7 cells, tamoxifen had little effect at 1ng/ml dose. Ad-mda7 reduced signal compared to Ad-luc. However when tamoxifen was combined with Ad-mda7, a supra-additive effect was observed, again demonstrating the enhanced effects of combining a chemotherapeutic agent with Ad-mda7.

2. Combination with Adriamycin

Ad-mda7 has been combined with adriamycin and evaluated for anti-tumor effects in breast cancer cell lines (FIG. 13). The graphs demonstrate that combining these two agents provides superior anti-tumor activity compared to either agent alone.

EXAMPLE 7: ACTIVATION OF CASPASE CASCADE BY AD-MDA7

1. Material and Methods

a. Cell Culture

Human non-small cell lung carcinoma cells A549, H460, H1299, human prostate cancer cells DU145, and human breast cancer cells MCF-7 were obtained from the American Type Culture Collection (ATCC, Bethesda, MD). All cells were maintained in DMEM medium containing 10% of Fetal Bovine Serum, antibiotics and L-glutamine. Normal human bronchial epithelium cells (NHBE cells) were obtained from Clonetics Inc (Clonetics Inc., Walkersville, MD) and maintained according to the manufacturer's instructions.

The cells were verified to be free of mycoplasma and used in the log phase of growth. Cells were routinely harvested with 0.125% Trypsin –1.3 mM EDTA (GIBCO).

b. Construction of Recombinant Adenoviral Vector

Same as described above.

c. Determination of Cell Growth Rate

Cancer or normal cell lines used in this study were plated in 12-well dishes with 2 x 10^4 cells in each well. Cells were infected with Ad-mda7, with Ad-Luc controls (5000 viral particles/cell), or with PBS as an additional control. Cells were harvested by trypsinization, diluted with trypan blue (GIBCO) and the numbers of viable cells were counted on a hemocytometer. In addition, inhibition of cell growth was assayed by XTT assay as per the manufacturer's guidelines (Cell Proliferation Detection Kit II, Roche) or by H^3-thymidine assay.

d. Cell Cycle Analysis

Fluorescence-activated cell sorter analysis was performed as follows: cells (5×10^5/plate) were seeded on 10cm plates and infected with PBS, Ad-mda7 or Ad-Luc at 5000 vp/cell. Cells were harvested by trypsinization at designated times (24, 48, 72 hrs

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after infection) and washed twice with PBS. Cells were fixed with 70 % ethanol, washed with PBS twice and resusupended with 500 µl of PI solution (5 µg/ml PI and 10µg/ml RNase). Cells were analyzed using a FASCscan analyzer.

5   e.   **Detection of Apoptosis**

Tumor cells were seeded in chamber slides (Falcon) at a density of 1 x 10⁵ cells/chamber. Cells were transduced with Ad-mda7 or Ad-Luc vectors. At different days post-infection, cells were analyzed for apoptosis by Hoechst 33342 staining (Boehringer Mannheim) and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick 10   end labeling (TUNEL) staining with Terminal Transferase (Boehringer Mannheim).

         f.   **Immunohistochemical Staining**

Immunohistochemical staining was carried out on virus infected cells to determine MDA-7 protein expression. Briefly, cells (H1299, A549, H460, and NHBE) 15   were plated at a density of 1x10⁵ in chamber slides (Falcon) and infected with Ad-mda7 or Ad-Luc (5000 viral particles/cell). 48 hrs later, cells were washed with PBS and fixed in 4% formalin solution for two minutes. After blocking of endogenous peroxidase activity with 0.3% H_2O_2 in methanol for 30 minutes, cells were incubated with normal goat serum for 30 minutes at room temperature. Following incubation, slides were 20   treated with rabbit polyclonal anti-MDA-7 antibody (1: 5000 dilution) for 60 minutes. After 30 minutes incubation with anti-rabbit secondary antibody (provided with ABC kit, Vector) expression of MDA-7 in cells was detected with DAB by enhancement with avidin-biotin reaction ABC kit. The slides were counterstained with hematoxylin and then mounted with Aqua-mount (Lerner Labs, Pittsburgh, PA). Negative controls 25   included cells uninfected but subjected through all staining proceeded.

         g.   **Western Blotting Analysis**

Cells were harvested by trypsinization, washed with PBS and resuspended in 100µl of lysis buffer (62.5mM Tris-Hcl, 2% SDS, 10% glycerol, 4M Urea). Cell extracts 30   were homogenized with sonicator for 30 sec and after an hour incubation on ice, cell

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extracts were spun for 5min at 14000 rpm at 4 °C. Cell extracts were collected and stored in –70°C. Protein concentrations of all extracts were determined using the Bio-Rad protein determination kit (Bio-Rad). Each of 50μg protein samples were diluted into 20μl with lysis buffer and 5% of 2-Mercaptoethanol (Bio-Rad) and heated in a water bath at

5   95°C for 5min. Then protein extracts were separated on a 10% SDS-PAGE gel in a vertical slab gel electrophoresis cell (Bio-Rad). Proteins were transferred from gel to nitrocellulose membrane (Hybond-ECL membranes, Amarsham International, Little Chalfont, England). Proteins were blocked in a blocking solution (5% dry milk and 0.3% Tween 20 in PBS) for one hour at room temperature. Membranes were incubated with

10   primary antibody and then horse raddish peroxidase labeled secondary antibodies followed by application of Enhanced Chemiluminescence Western Blotting Detection System (Amersham) for 30 seconds. Proteins were visualized on Amersham Hyperfilm enhanced chemiluminescence film using exposure time varying 30 seconds to 30minutes.

15   **2.   Inhibition of Cell Proliferation by Overexpression of MDA-7**

To detect MDA-7 expression in cells, A549, H1299, H460, and NHBE cells were infected with 5000vp/cell of Ad-mda7. Forty-eight hours later cells were fixed and stained with anti-MDA-7 antibody. Uninfected cells were stained with the same antibody as controls. High level of MDA-7 expression was observed in cytoplasm of cells, while

20   no stained cells were seen in uninfected controls (FIG. 14).

A549, H1299, H460, and NHBE cells were prepared in 12 well plates and treated with Ad-mda7, Ad-Luc, or PBS. The numbers of viable cells were counted from day 1 to day 5 after treatment. Infection with Ad-mda7 significantly suppressed cell proliferation

25   in all the tumor cell lines as compare to PBS or Ad-Luc controls (Fig 23).

Cell cycle analysis using PI staining showed a G2/M cell cycle arrest in Ad-mda7-infected A549 and H1299 cells. In contrast, PBS and Ad-Luc infection did not affect the cell cycle (FIG. 14).

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Following Ad-mda7 infection, morphological changeswere observed in tumor cells. These changes, such as flattenning and enlargement were observed in all of infected cell lines. Apoptotic morphological changes were visualized using Hoechst

5   33342. 72 hours after infection of Ad-mda7 or Ad-Luc, nuclear condensation and fragmentation were observed in Ad-mda7 infected A549, H1299, and H460 cells, while apoptotic alterations were not seen in NHBE cells. TUNEL staining demonstrated many positive cells in Ad-mda7 infected A549 cells, while very few positive cells were seen in NHBE cells. TUNEL positive cells were also very rare in Ad-luc treated samples.

10

These results showed significant supression of cell proliferation with concomitant G2/M cell cycle arrest and induction of apoptosis in lung cancer cell lines. In contrast, in NHBE cells overexpression of MDA-7 resulted in minimal suppression of cell proliferation, but did not induce apoptosis.

15

3.   Upregulation of p53 and Bax in Cells with Wild type p53

Cells were infected with Ad-mda7 and Ad-Luc, and cell extracts were harvested at 24, 48, and 72 hours after infection for Western blot analysis. Cell extracts from untreated cell were harvested as a control. MDA-7 protein expression was detected in all

20   of the Ad-mda7-infected cancer cell lines. Untreated controls and Ad-Luc-infected cells did not show any expression of MDA-7 protein. Upregulation of p53 protein was seen in p53 wild type A549 and H460 cells after Ad-mda7 infection. As predicted, no expression or modulation of p53 was seen in p53-deleted H1299 cells. An increase in BAX protein levels was demonstrated in A549 and H460 cells (p53 wild-type), while no change was

25   observed in H1299 (p53-null) cells. The expression level of Bcl-2 was not changed in all of the three cell lines analyzed. In the Bax-deficient, human prostate cancer cell line DU145, p53 expression levels were not changed and BAX was not detected. However, DU-145 cells were sensitive to Ad-mda7 infection and displayed growth arrest and apoptosis. p53 and bax are up-regulated by Ad-mda7 in p53 wild type tumor cells. In

addition, caspases 3 and 9 and PARP are activated by Ad-mda7. Normal cells do not exhibit alterations in apoptotic mediators.

4.   Activation of Caspase Cascade and Cleavage of PARP

5   Western blots demonstrated activation of the caspase cascade by Ad-mda7 infection (FIG. 14B). The proforms of caspase-9 and caspase-3 were cleaved and converted to the activated/ cleaved forms 48 hrs after Ad-mda7 infection in A549 and H460 cells and after 72hrs in H1299 cells. Cleavage of caspase-8 was demonstrated after 48 hrs of Ad-mda7 infection in A549 and H460 cells. Poly (ADP-ribose) polymerase

10   (PARP) was cleaved in A549 and H460 cells after 48 hrs in H1299 cells. In Bax-deficient DU145 cells, caspase-9 and caspase-3 were cleaved after 72 hrs of Ad-mda7 infection.

EXAMPLE 8: *IN VIVO* EFFECTS OF AD-MDA7

15

1.   Materials and Methods

A.   Cell culture

Human non-small cell lung carcinoma cells A549 and H1299 were obtained from the American Type Culture Collection (ATCC, Bethesda, MD). All cells were

20   maintained in RPMI1640 medium containing 10% of Fetal Bovine Serum, antibiotics and L-glutamine. Prior to start of the experiments, the cells were verified to be free of mycoplasma and used in the log phase of growth. Cells were routinely harvested with 0.125% Trypsin –1.3 mM EDTA (GIBCO).

25   **B.   Construction of recombinant adenoviral vector**

Replication-deficient human type 5 Adenoviral vectors (Ad5) carrying the mda-7 or Luc genes linked to an internal CMV-IE promoter and followed by SV40 polyadenylation (pA) signal have been constructed and will be referred to as Ad-mda7

and Ad-luc, respectively. Viruses were propagated in 293 cells and purified by chromatography.

C.   Apoptotic cell staining

5   Sections were stained for apoptotic cell death using the terminal deoxynucleotide transferase (Tdt) (Boehringer Mannheim) kit and counterstained with methylene blue or methlene green as described (Fujiwara *et al.*, 1994).

D.   Western blotting analysis

10   Western blotting was performed as described above. Cells were harvested by trypsinization, washed with PBS and resuspended in 100 µl of lysis buffer (62.5 mM Tris-Hcl, 2% SDS, 10% glycerol, 4 M Urea). Cell extracts were homogenized with sonicator for 30sec and after an hour incubation on ice, cell extracts were spun for 5min at 14000 rpm at 4 °C. Cell extracts were collected and stored in -70°C. Protein

15   concentrations of all extracts were determined using the Bio-Rad protein determination kit (Bio-Rad). Each of 50 µg protein samples were diluted into 20 µl with lysis buffer and 5% of 2-Mercapto Ethanol (Bio-Rad), and heated in a water bath at 95°C for 5min. Then protein extracts were separated on a 10% SDS-PAGE gel in a dual vertical slab gel electrophoresis cell (Bio-Rad).

20

Proteins were transferred from gel to nitrocellulose membrane (Hybond-ECL membranes). Proteins were blocked in a blocking solution (5% dry milk and 0.3% Tween 20 in PBS) for 1 hour at room temperature. Then membranes were incubated with primary antibody. Horse raddish peroxidase labeled secondary antibodies were applied

25   and Enhanced chemiluminescence Western Blotting detection system (Amersham) was applied for 30 second and proteins were then visualizen on Amersham Hyperfilm enhanced chemiluminescence film using exposure time varying 30sec to 30min.

E.   Evaluation of tumor growth and treatments *in vivo*

Prior to the start of all experiments involving subcutaneous tumor growth and treatments, *nu/nu* mice were irradiated (3.5 Gy) using a cesium source to enhance tumor uptake. In all the experiments, $5x10^6$ tumor cells (H1299, A549) suspended in 100 μl sterile phosphate buffered saline (PBS) were injected into the right dorsal flank. When

5   the tumor had reached a size of 50-100mm^3, animals were randomized into three groups (n = 8 animals/group) and treatment initiated as follows. Group 1 received no treatment, Group 2 received Ad-Luc (5x109 vp / dose) and Group 3 received Ad-mda-7 (5x109 vp / dose) every alternate day for a total of three doses. Intratumoral injections were performed under anesthesia using methoxyflurane (Schering Plough, Kenilworth, NJ) as

10   per institutional guidelines. Tumor measurements were recorded every other day without knowledge of the treatment groups, and the volume was calculated using the formula V (mm3) = a x b^2 /2, where "a" is the largest dimension and "b" is the perpendicular diameter. Antitumor efficacy data are presented as cumulative tumor volumes for all animals in each group to account for both size and number of tumors.

15

F.   Immunohistochemical analysis

Tumors established subcutaneously in nude mice were obtained and fixed in 10% buffered formalin, paraffin embedded and cut as 4 μm thick sections. Sections were stained for mda-7 gene expression. Briefly, tissue sections were treated with 0.3% H$_2$O$_2$

20   in methanol for 30 minutes to block endogenous peroxidase activity and were subsequently incubated with normal goat serum for 30 minutes at room temperature. Following incubation, slides were treated with rabbit polyclonal anti-MDA-7 antibody (1: 5000 dilution) for 60 minutes. After 30 minutes incubation with anti-rabbit secondary antibody (provided with ABC kit, Vector) protein expression of MDA-7 in tissues were

25   detected with DAB by enhancement with avidin-biotin reaction ABC kit. The slides will be counterstained with hematoxylin and then mounted with Aqua-mount (Lerner Labs., Pittsburgh, PA). The number of tumor cells staining positive for MDA-7 were analyzed under bright field microscopy and quantitated in a blind fashion using image analysis and statpro software. A total of at least five fields per specimen were analyzed.

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G.   TUNEL staining

Tissue sections obtained from subcutaneous tumors were stained for apoptotic cell death using the terminal deoxynucleotide transferase kit (Tdt) (Boehringer Mannheim). In all the staining procedures, appropriate negative controls were included. The number of tumor cells staining TUNEL positive were analyzed under bright field microscopy and quantitated in a blind fashion using image analysis and statpro software. A total of at least five fields per specimen were analyzed.

H.   Statistical analysis

The statistical significance of the experimental results was calculated using Student's t-test for tumor measurements.

2.   *In vivo* suppression of local tumor growth by Ad-mda7

The therapeutic effect of the mda–7 gene on H1299 and A549 subcutaneous tumors was evaluated in nude mice. Mice bearing each tumor cell type (H1299 and A549) were divided into three groups, one receiving no treatment, one treatment with Ad-Luc, and one treatment with the Ad-mda-7 daily for a total of three doses (5×10^9 viral particles/dose). A significant growth inhibition of H1299 tumors and A549 tumors was observed in mice treated with the Ad-mda-7 compared with the tumor growth in the two control groups for each tumor type.

Further evidence that the observed therapeutic effect was due to mda-7 gene expression was obtained by removing subcutaneous tumors 48 hours after injection and analyzing them by immunohistochemistry. mda-7 gene expression was observed in tumor cells in animals receiving the Ad-mda7, as compared to no positive staining in control tumors that were either not treated or treated with Ad-Luc.

MDA-7 gene expression *in situ* results in apoptotic cell death through caspase-3 and Apo2/TRAIL activation. To understand the mechanism of tumor inhibition mediated by mda-7, subcutaneous tumors harvested at 48 hours following the last treatment were analyzed for apoptotic tumor cell death by TUNEL staining. Tumors from control mice that were either untreated or treated with Ad-Luc showed minimal apoptotic cell death while tumors from animals treated with Ad-mda-7 demonstrated extensive apoptosis.

Since apoptosis is mediated by activation of caspases, tumor tissues were examined for caspase-3, a downstream caspase. Activated form of caspase-3 was observed in tissues treated with Ad-mda-7 while no caspase-3 activation was observed in the tissues from control mice. Similarly, activation of Apo2/TRAIL was observed in tumors expressing mda-7. In contrast, TRAIL expression was not observed in tumors that were not treated or treated with Ad-luc.

3.   **MDA-7 Expression Results in Upregulation of Costimulatory Molecules**

The ability of dying tumor cells *in situ* to activate costimulatory molecules, B7 and ICAM, was investigated. Subcutaneous tumors injected with Ad-MDA7 or Ad-Luc were harvested 48 hrs following the last dose and analyzed by immunohistochemistry. Expression of B7 (7.1 and 7.2) and ICAM was observed in tumors expressing MDA-7 while no expression was observed in tumors treated with Ad-Luc.

4.   **Expression of MDA-7 in *in situ* Tumor Inhibits Angiogenesis**

To further determine the tumor suppressive effects of mda-7, subcutaneous tumors were analyzed for CD31 expression, a marker frequently used to identify angiogenesis in tumors. Subcutaneous tumors treated with Ad-mda-7 demonstrated fewer numbers of blood vessels when compared to tumors treated with Ad-luc or no treatment groups.

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EXAMPLE 9: EFFICACY OF AD-MDA7 TO PREVENT METASTATIC SPREAD OF TUMOR

Experiments have demonstrated that Ad-mda7 can inhibit metastatic spread of lung cancer tumors *in vivo*. Further experiments will be performed using melanoma cell

5   lines to evaluate the ability of MDA-7 to prevent the metastatic spread of melanoma tumors. Techniques and protocols discussed previously will be employed.

Human melanoma xenografts will be established by subcutaneous injection of human melanoma cells (1 x 10^6 cells) into the flanks of nude mice. TXM-1 or TXM-18

10   cells may be used. Once the tumor reaches 5 mm mean diameter, increasing doses of Ad-mda7 or control Ad-luc will be injected into the tumors. Doses of 3 x 10^7 to 3 x 10^9 pfu will be tried. Adenoviral vector will be delivered in three injections of approximately 33 ml, total 100 ml, intralesionally. Each injection will be orthogonally oriented to the preceding injection to ensure efficient tumor coverage. After establishment of the

15   appropriate dose, tumor xenografts will be treated with a single 100 ml dose or multiple fractional doses equaling 100 ml over a three day time period to assess the effectiveness of the described administration regimens. Following these studies, a comparison between single dose administration versus multiple dose administration will be performed, with a dose being defined as 100 ml injection of the previously optimized concentrations in

20   pfus. Efficacy studies will consist of the treatment of tumor xenografts following the established adenoviral concentrations and treatment regimen for 3 to 5 days. Efficacy will be assessed by the reduction in tumor size. Tumor size will be determined by the direct measurement of tumor diameters.

25   Ad-mda7 treated tumors will be evaluated for   expression of MDA-7 protein and apoptosis induction. Immunohistochemical detection of MDA-7 and TUNEL assay detection of apoptosis will be used to evaluate the efficacy of Ad-mda7 treatment at the cellular level. An MDA-7 antibody that specifically recognizes MDA-7 protein will be employed for immunohistochemistry procedures. Endothelial cells in the melanoma

30   xenografts will be detected with antibodies directed against mouse CD-31. Areas of the

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tumor sections with high numbers of capiallaries and small venules will be found by scanning the sections at low power (x40 and x100). In these areas individual vessels will be counted in x200 magnification fields, and average scores recorded for the treated and untreated tumor samples. This method has been used to compare blood distribution and

5   density in human xenografts in nude mice (Yoneda *et al.*, 1998).

EXAMPLE 10: MODULATION OF GROWTH FACTORS DURING
ECTOPIC EXPRESSION OF MDA-7

10   Because it has been hypothesized that MDA-7 has an autocrine/paracrine activity, the effect of Ad-mda7 on melanoma cells will be evaluated with respect to the secretion of factors involved in the progression of melanoma. ELISA assays will be used to address the release of these soluble mediators, such as different types of TGF-β1, IL-8, IL-10, and bFGF. Melanoma cells lines and normal cells will be treated with Ad-mda7,

15   Ad-luc, or diluent control and then monitored for modulation of growth factor levels in culture supernatant after 24-48 hours. Immunoblotting on the lysates may also be performed at various times post-treatment.

EXAMPLE 11: AD-MDA7 ENHANCES ACTIVITY OF HERCEPTIN

20
The breast cancer SkBr3 (Her2+) and MCF-7 (Her2-) cell lines were both obtained from ATCC. Cells were plated at a density of 1000 cells/well in Nunc 2-chamber slides and propagated in DMEM medium with 10% FBS. The following day, the cells were left untreated or treated with Ad-mda7 at (increasing MOIs: 0, 500, 1000

25   and 2000 vp/cell) without (M series) or with Herceptin (M+H series) at a final concentration of 1 µg/mL. The cells were washed after 3 hours and growth media (with or without Herceptin, as indicated) was replaced. Three days later viable cells were counted using the trypan blue exclusion assay (average of 3-4 fields) and plotted as shown in FIG. 15. Herceptin alone yields approximately 12% dead cells in both cell

lines. However, Ad-mda7 appeared to enhance the killing effect of Herceptin in breast cancer cell lines.

* * * * * * * * * * * * * * * * * *

5

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied

10 to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and

15 modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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WHAT IS CLAIMED IS:

1.   A method for treating a patient with a hyperproliferative disease comprising administering to the patient an effective amount of an expression cassette comprising a nucleic acid sequence encoding a human MDA-7 polypeptide under the control of a promoter operable in eukaryotic cells.

2.   The method of claim 1, wherein the hyperproliferative disease is further defined as cancer.

3.   The method of claim 2, wherein the cancer comprises a tumor.

4.   The method of claim 2, wherein the cancer is melanoma, non-small cell lung, small-cell lung, lung, hepatocarcinoma, retinoblastoma, astrocytoma, glioblastoma, gum, tongue, leukemia, neuroblastoma, head, neck, breast, pancreatic, prostate, renal, bone, testicular, ovarian, mesothelioma, cervical, gastrointestinal, lymphoma, brain, colon, or bladder.

5.   The method of claim 1, wherein the hyperproliferative disease is rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, leiomyomas, adenomas, lipomas, hemangiomas, fibromas, vascular occlusion, restenosis, atherosclerosis, pre-neoplastic lesions, carcinoma in situ, oral hairy leukoplakia, or psoriasis.

6.   The method of claim 1, wherein the expression cassette is an expression vector.

7.   The method of claim 6, wherein the expression vector is a viral vector.

8.   The method of claim 7, wherein the viral vector is administered at between about 10^3 and about 10^{13} viral particles.

9.   The method of claim 7, wherein the viral vector is an adenoviral vector, a retroviral vector, a vaccinia viral vector, an adeno-associated viral vector, a polyoma viral vector or a herpesviral vector.

5   10.   The method of claim 7, wherein the viral vector is an adenoviral vector.

11.   The method of claim 1, wherein the promoter is CMV IE, dectin-1, dectin-2, human CD11c, F4/80, SM22, or MHC class II promoter.

10   12.   The method of claim 1, wherein the expression cassette is administered to the patient in a lipoplex.

13.   The method of claim 12, wherein the lipoplex comprises DOTAP and at least one cholesterol, cholesterol derivative, or cholesterol mixture.

15

14.   The method of claim 1, wherein administering is by injection of the expression cassette.

15.   The method of claim 14, wherein administering comprises multiple injections.

20

16.   The method of claim 14, wherein the injection is local, regional, or distal to the disease or a tumor site.

17.   The method of claim 1, wherein administering is via continuous infusion, 25   intratumoral injection, or intravenous injection.

18.   The method of claim 3, wherein the expression cassette is administered to the tumor bed prior to or after resection of the tumor.

19.   The method of claim 3, wherein the expression cassette is administered to the tumor bed both prior to and after tumor resection.

20.   The method of claim 1, wherein the patient is a human.

5

21.   The method of claim 1, wherein the nucleic acid sequence encodes a full-length MDA-7 polypeptide.

22.   The method of claim 1, wherein the nucleic acid sequence encodes amino acids from about 182 to about 206 of SEQ ID NO:2.

10

23.   The method of claim 1, wherein the nucleic acid sequence encodes amino acids from about 175 to about 206 of SEQ ID NO:2.

15   24.   The method of claim 1, wherein the nucleic acid sequence encodes amino acids from about 150 to about 206 of SEQ ID NO:1.

25.   The method of claim 1, wherein the nucleic acid sequence encodes amino acids from about 100 to about 206 of SEQ ID NO:2.

20

26.   The method of claim 1, wherein the nucleic acid sequence encodes amino acids from about 49 to about 206 of SEQ ID NO:2.

27.   The method of claim 1, wherein the MDA-7 polypeptide is lacking a signal
25   sequence from the full-length MDA-7 polypeptide sequence.

28.   The method of claim 27, wherein the expression cassette further comprises a second nucleic acid sequence encoding a heterologous secretory signal.

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29.   The method of claim 28, wherein the secretory signal is further defined as a positively charged N-terminal region in combination with a hydrophobic core.

30.   A method of treating a patient with cancer comprising

5   a)   administering to the patient an effective amount of an expression cassette comprising a nucleic acid sequence encoding a human MDA-7 polypeptide under the control of a promoter operable in eukaryotic cells; and

       b)   administering at least one other anticancer treatment.

10

31.   The method of claim 30, wherein the anticancer treatment is chemotherapy, immunotherapy, surgery, radiotherapy, gene therapy with a second therapeutic polynucleotide other than a polynucleotide encoding the MDA-7 polypeptide, or other biotherapy.

15

32.   The method of claim 31, wherein the expression cassette is administered to the patient before, during, or after the other anti-cancer treatment.

33.   The method of claim 30, wherein the expression cassette encodes a full-length

20   human MDA-7 protein.

34.   The method of claim 30, wherein the expression cassette further encodes a secretory signal.

25   35.   The method of claim 30, wherein the cancer further comprises a tumor.

36.   The method of claim 30, wherein the cancer is melanoma, non-small cell lung, small-cell lung, lung, hepatocarcinoma, retinoblastoma, astrocytoma, glioblastoma, leukemia, neuroblastoma, head, neck, breast, pancreatic, prostate, renal, bone, testicular,

30   ovarian, mesothelioma, cervical, gastrointestinal, lymphoma, brain, colon or bladder.

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37.   The method of claim 30, wherein the expression cassette is an adenovirus vector.

38.   The method of claim 37, wherein the adenovirus vector is administered at
between about 10^3 and about 10^{15} viral particles.

39.   The method of claim 30, wherein the expression cassette is administered to the
patient in a lipoplex.

40.   The method of claim 39, wherein the lipoplex comprises DOTAP and at least one
cholesterol, cholesterol derivative, or cholesterol mixture.

41.   The method of claim 30, wherein administering is by injection of the expression
cassette.

42.   The method of claim 41, wherein the injection is local, regional, or distal to the
cancer.

43.   The method of claim 30, wherein administering is via continuous infusion,
intratumoral injection, or intravenous injection.

44.   The method of claim 30, wherein the patient is a human.

45.   An expression vector encoding a mda-7 coding region under the control of a
promoter operable in an eukaryotic cell, wherein the coding region contains a deletion
corresponding to N-terminal sequences.

46.   The expression vector of claim 45, wherein the expression vector lacks coding
sequences corresponding to amino acid 1 to about amino acid 49 of SEQ ID NO:2.

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47.   The expression vector of claim 45, wherein the expression vector lacks coding sequences corresponding to amino acid 1 to about amino acid 100 of SEQ ID NO:2.

48.   The expression vector of claim 45, wherein the expression vector lacks coding sequences corresponding to amino acid 1 to about amino acid 150 of SEQ ID NO:2.

49.   The expression vector of claim 45, wherein the expression vector lacks coding sequences corresponding to amino acid 1 to about amino acid 182 of SEQ ID NO:2.

50.   The expression vector of claim 45, wherein the expression vector further encodes a secretory signal.

51.   The expression vector of claim 45, wherein the promoter is CMV IE, dectin-1, dectin-2, human CD11c, F4/80, SM22, or MHC class II promoter.

52.   The expression vector of claim 45, wherein the expression vector is further defined as a viral vector.

53.   The expression vector of claim 52, wherein the viral vector is an adenovirus vector, a retrovirus vector, a vaccinia virus vector, an adeno-associated virus vector, a polyoma virus vector, or a herpesvirus vector.

54.   The expression vector of claim 53, wherein the viral vector is an adenovirus vector.

55.   A method of treating a hyperproliferative disorder comprising admininstering to a hyperproliferative cell an effective amount of a nucleic acid molecule encoding MDA-7 in combination with chemotherapy, immunotherapy, surgery, radiotherapy, or gene therapy with a second therapeutic polynucleotide other than a polynucleotide encoding an MDA-7 polypeptide.

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56.   The method of claim 55, wherein the chemotherapy, immunotherapy, surgery, radiotherapy, or gene therapy with a second therapeutic polynucleotide other than a polynucleotide encoding an MDA-7 polypeptide is administered prior to the nucleic acid molecule encoding MDA-7.

57.   The method of claim 55 wherein the chemotherapy, immunotherapy, surgery, radiotherapy, or gene therapy with a second therapeutic polynucleotide other than a polynucleotide encoding an MDA-7 polypeptide is administered after the nucleic acid molecule encoding MDA-7.

58.   The method of claim 55, wherein the chemotherapy comprises a DNA damaging agent.

59.   The method of claim 58, wherein the DNA damaging agent is gamma-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), or hydrogen peroxide.

60.   The method of claim 59, wherein the DNA damaging agent is adriamycin.

61.   The method of claim 55, wherein the chemotherapy comprises a cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, taxotere, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin, methotrexate, or any analog or derivative variant thereof.

62.   The method of claim 61, wherein the chemotherapy comprises tamoxifen.

63.   The method of claim 61, wherein the chemotherapy comprises taxotere.

64.   The method of claim 55, wherein immunotherapy comprises Herceptin.

5   65.   The method of claim 55, wherein the nucleic acid is comprised within a viral vector.

66.   The method of claim 55, wherein the nucleic acid is comprised in a lipid composition.

10

67.   The method of claim 55, wherein the hyperproliferative disorder is cancer.

68.   The method of claim 67, wherein the cancer is melanoma, non-small cell lung, small-cell lung, lung, hepatocarcinoma, retinoblastoma, astrocytoma, glioblastoma,

15   leukemia, neuroblastoma, head, neck, breast, pancreatic, prostate, renal, bone, testicular, ovarian, mesothelioma, cervical, gastrointestinal, lymphoma, brain, colon or bladder.

69.   The method of claim 55, wherein the hyperproliferative disorder is vascular occlusion, restenosis, or rheumatoid arthritis.

20

70.   A method for treating a patient with a hyperproliferative disease comprising administering to the patient an amount of an adenovirus composition effective to confer a therapeutic benefit on the patient, wherein the adenovirus composition comprises an adenovirus vector construct comprising an mda-7 gene under the control of a promoter.

25

71.   The method of claim 70, wherein the adenovirus composition is dispersed in a pharmacologically acceptable solution.

72.   The method of claim 71, wherein the pharmacologically acceptable solution

30   comprises a lipid.

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73.   The method of claim 70, wherein the adenovirus composition is administered to the patient intravenously, intraperitoneally, intratracheally, intratumorally, intramuscularly, endoscopically, intralesionally, percutaneously, subcutaneously, regionally, or by direct injection or perfusion.

74.   The method of claim 70, wherein from about 10^3 to about 10^{15} viral particles are administered to the patient.

75.   The method of claim 74, wherein from about 10^5 to about 10^{12} viral particles are administered to the patient.

76.   The method of claim 74, wherein from about 10^7 to about 10^{10} viral particles are administered to the patient.

77.   The method of claim 70, wherein the patient is administered the adenovirus composition more than once.

78.   A method of inducing apoptosis in a cancer cell comprising administering to the cancer cell in a subject an expression cassette comprising a nucleic acid sequence encoding a human MDA-7 protein under the control of a promoter operable in eukaryotic cells.

79.   The method of claim 78, wherein the MDA-7 protein is truncated.

80.   A method of inducing apoptosis in a cancer cell comprising administering to a noncancerous cell in a subject an expression cassette comprising a nucleic acid sequence encoding a human MDA-7 protein under the control of a promoter operable in eukaryotic cells, wherein the MDA-7 protein is expressed and secreted.

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81.   The method of claim 80, wherein the MDA-7 protein is truncated.

82.   The method of claim 80, wherein the noncancerous cell is adjacent to the cancer cell.

5

83.   A method for treating a patient with cancer comprising administering to a noncancerous cell in the patient an effective amount of an adenovirus composition to confer a therapeutic benefit on the patient, wherein the adenovirus composition comprises an adenovirus vector construct comprising a mda-7 gene under the control of a promoter.

10

84.   The method of claim 83, wherein the mda-7 gene is truncated.

85.   A method of treating a tumor by inducing apoptosis in transfected and untransfected tumor cells comprising administering to the tumor an adenovirus

15   composition comprising an adenovirus vector construct comprising a human mda-7 gene under the control of a promoter, wherein the transfected cells express and secrete a truncated MDA-7 polypeptide.

86.   The method of claim 85, wherein the human mda-7 gene is truncated.

20

87.   The method of claim 86, wherein the human mda-7 gene comprises fewer than 700 contiguous nucleotides from SEQ ID NO:1.

88.   A method of treating cancer comprising administering to a subject with cancer an

25   adenovirus composition comprising an adenovirus vector construct comprising a human mda-7 gene under the control of a promoter to a cell that does not have mutated p53, Rb, ras, or p16 genes, in an amount effective to induce apoptosis in a cell that does have a mutated p53, Rb, ras, or p16 gene.

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89.   A method of treating a subject with a tumor comprising administering to the subject a nucleic acid molecule comprising a human mda-7 gene under the control of a promoter in an amount effective to inhibit angiogenesis around the tumor.

5   90.   The method of claim 89, wherein the nucleic acid molecule is an adenoviral vector.

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ABSTRACT

The present invention relates to gene therapy methods for the treatment of human disease. More specifically, the invention is directed, in one embodiment, to methods for

5   treating a subject with a hyperproliferative disease. In another embodiment, an adenoviral expression construct comprising a nucleic acid encoding a human MDA-7 protein under the control of a promoter operable in eukaryotic cells is administered to the patient with a hyperproliferative disease. The present invention thus provides a gene therapy for treating hyperproliferative disease by elevating the expression of MDA-7

10   resulting in inhibition of cell growth and induction of apoptosis in hyperproliferative cells.

SEQUENCE LISTING

<110> MHASHILKAR, ABNER
       SCHROCK, BOB
       CHADA, SUNIL

<120> METHODS FOR TREATMENT OF HYPERPROLIFERATIVE DISEASES
       USING HUMAN MDA-7

<130> INGN:090US

<140> UNKNOWN
<141> 2000-07-13

<150> 60/200,768
<151> 2000-04-28

<160> 60/144,354
<161> 1999-07-15

<170> 2

<180> PatentIn Ver. 2.0

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gcaaagcctg tggactttag ccagaccctt ctgccctcct ttgctggcga cagcctctca 180
aatgcagatg gttgtgctcc cttgcctggg ttttaccctg cttctctgga gccaggtatc 240
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caggcggttt ctgctattcc ggagagcatt caaacagttg gacgtagaag cagctctgac 660
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<211> 206
<212> PRT
<213> Human

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  1   5   10   15

Phe Cys Pro Pro Leu Leu Ala Thr Ala Ser Gln Met Gln Met Val Val
                 20   25   30

Leu Pro Cys Leu Gly Phe Thr Leu Leu Leu Trp Ser Gln Val Ser Gly
             35   40   45

Ala Gln Gly Gln Glu Phe His Phe Gly Pro Cys Gln Val Lys Gly Val

1

Val Pro Gln Lys Leu Trp Glu Ala Phe Trp Ala Val Lys Asp Thr Met
 65   70   75   80

Gln Ala Gln Asp Asn Ile Thr Ser Ala Arg Leu Leu Gln Gln Glu Val
             85   90   95

Leu Gln Asn Val Ser Asp Ala Glu Ser Cys Tyr Leu Val His Thr Leu
             100   105   110

Leu Glu Phe Tyr Leu Lys Thr Val Phe Lys Asn Tyr His Asn Arg Thr
         115   120   125

Val Glu Val Arg Thr Leu Lys Ser Phe Ser Thr Leu Ala Asn Asn Phe
     130   135   140

Val Leu Ile Val Ser Gln Leu Gln Pro Ser Gln Glu Asn Glu Met Phe
145   150   155   160

Ser Ile Arg Asp Ser Ala His Arg Arg Phe Leu Leu Phe Arg Arg Ala
             165   170   175

Phe Lys Gln Leu Asp Val Glu Ala Ala Leu Thr Lys Ala Leu Gly Glu
         180   185   190

Val Asp Ile Leu Leu Thr Trp Met Gln Lys Phe Tyr Lys Leu
     195   200   205

2
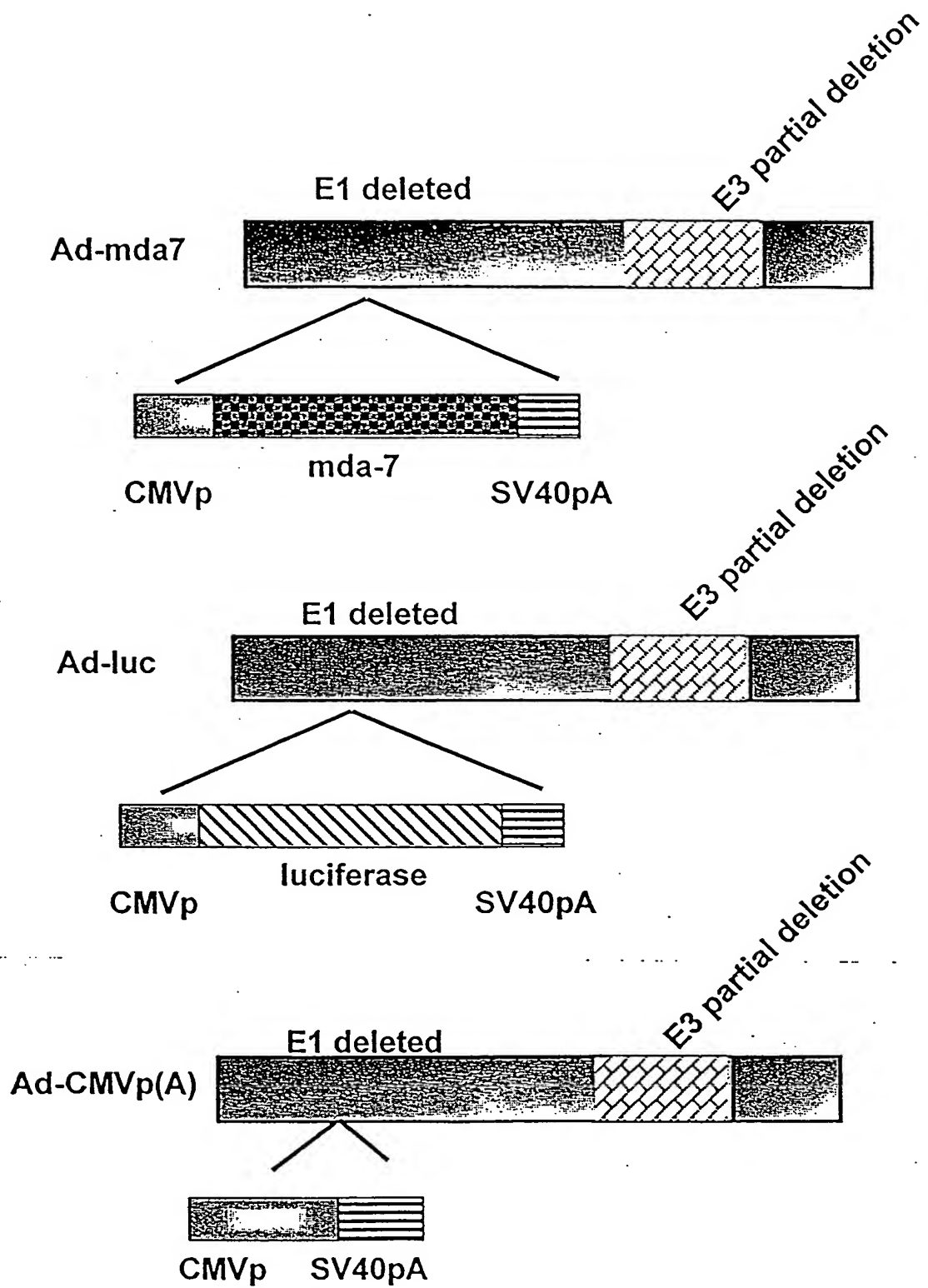
FIG. 1

FIG. 2

A   T47D cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)
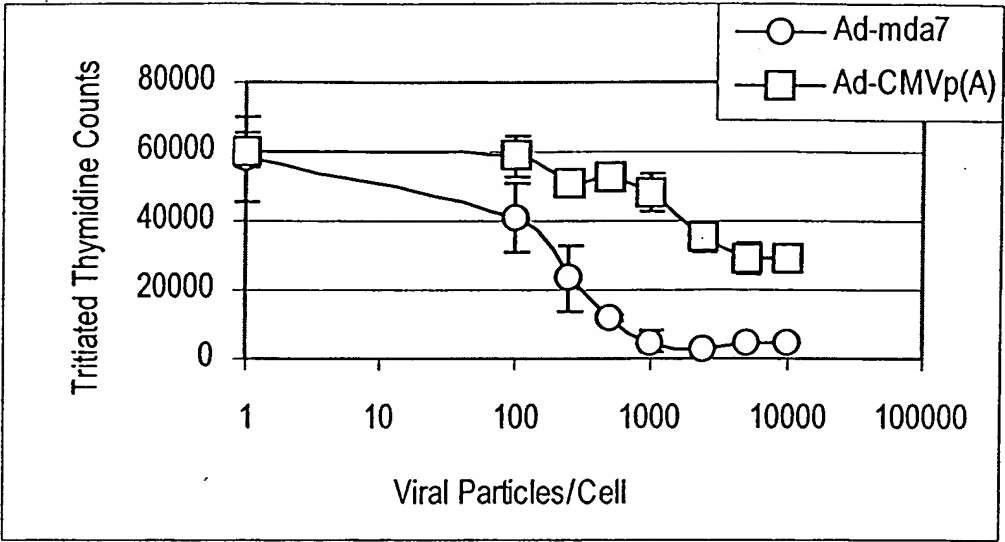
B   MCF-7 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)
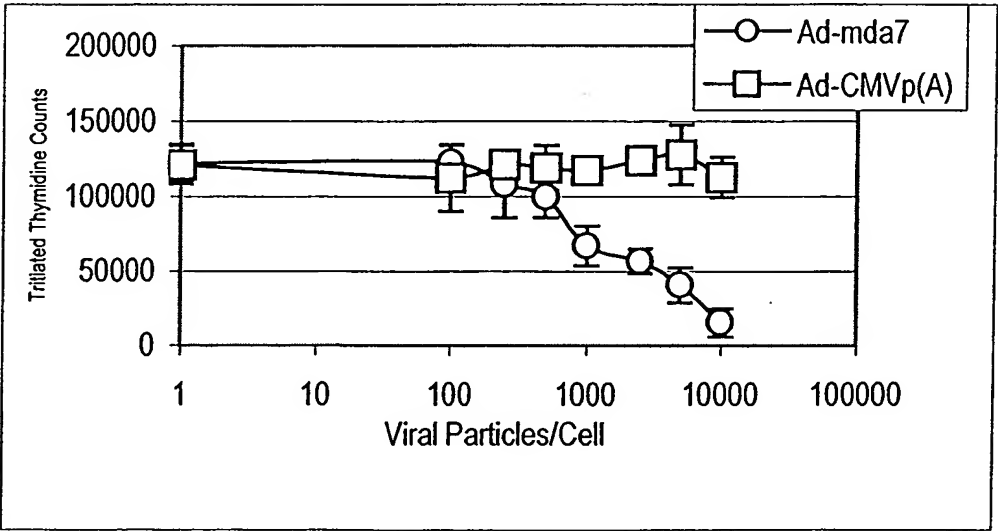
FIG. 3

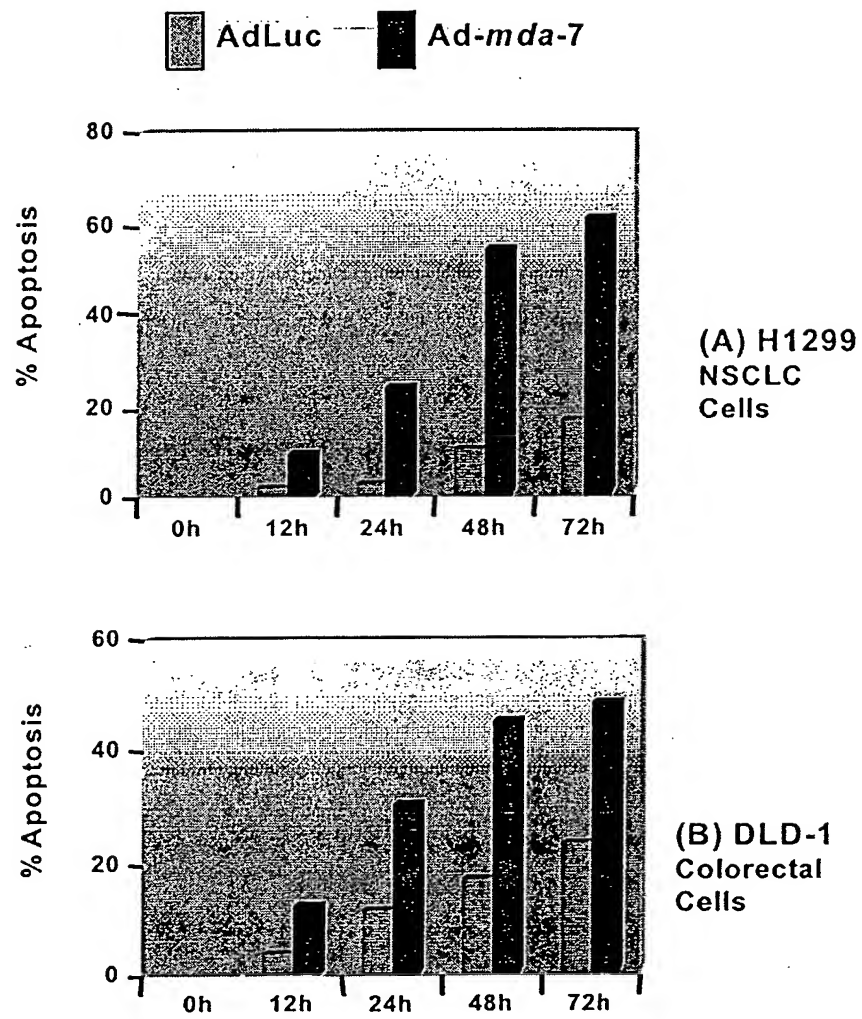A   MDA-MB-361 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)
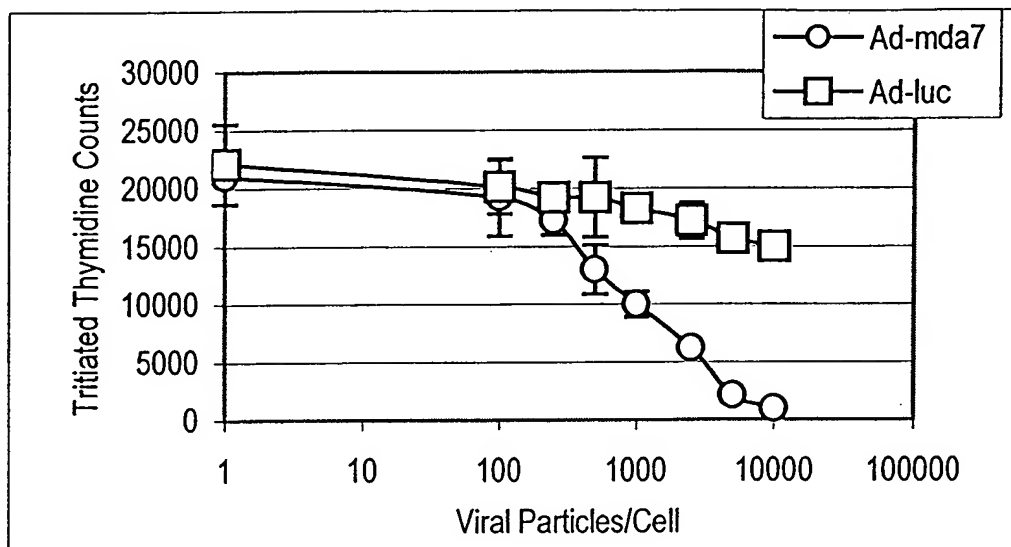
B   BT-20 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)
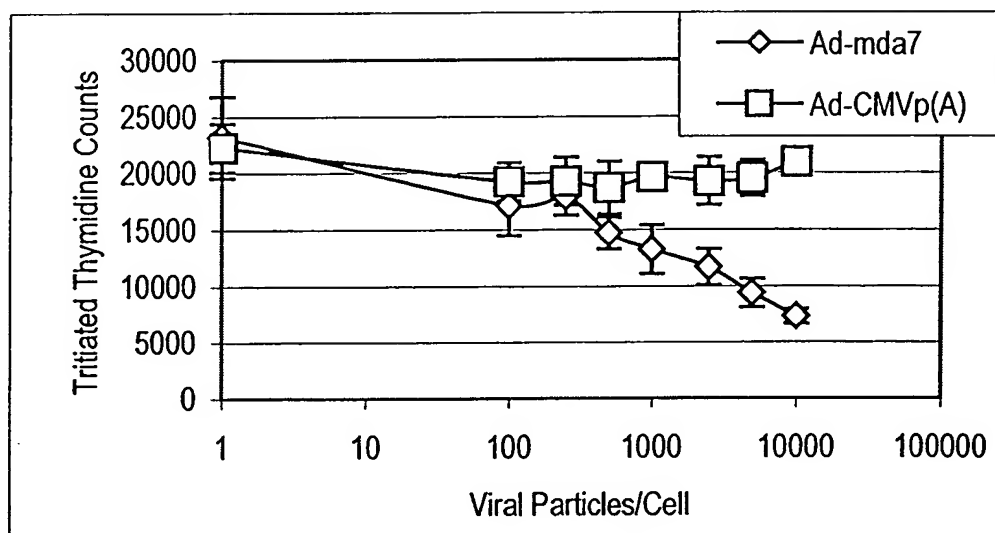
FIG. 4

A   H1299 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)
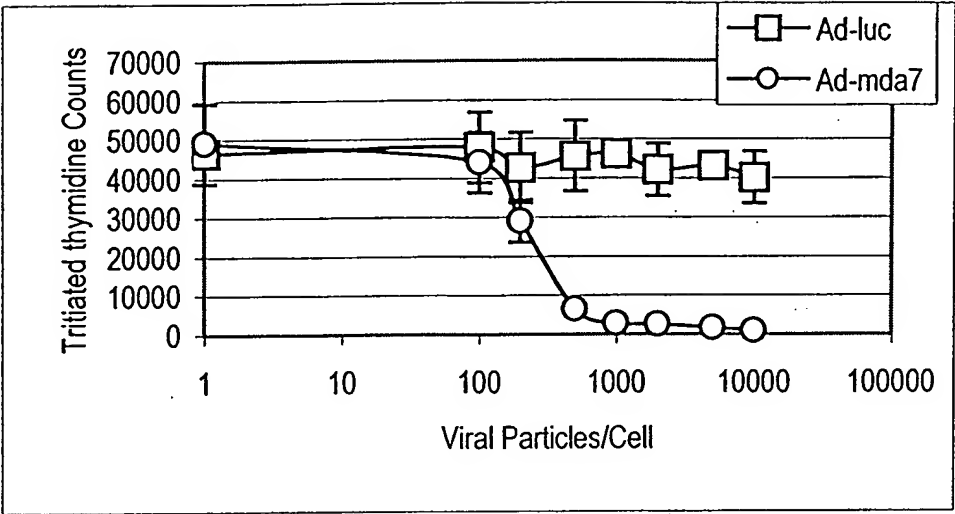
B   H322 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)
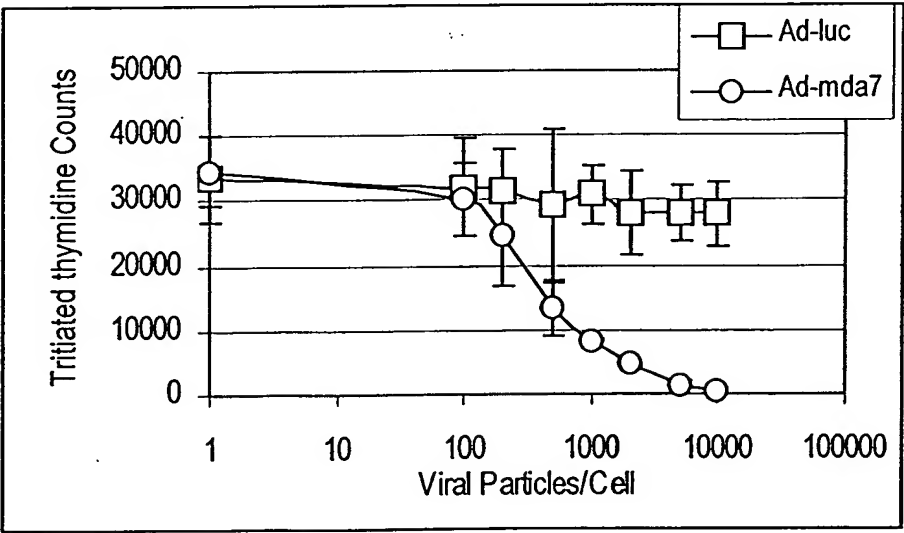
FIG. 5

A   SW620 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)
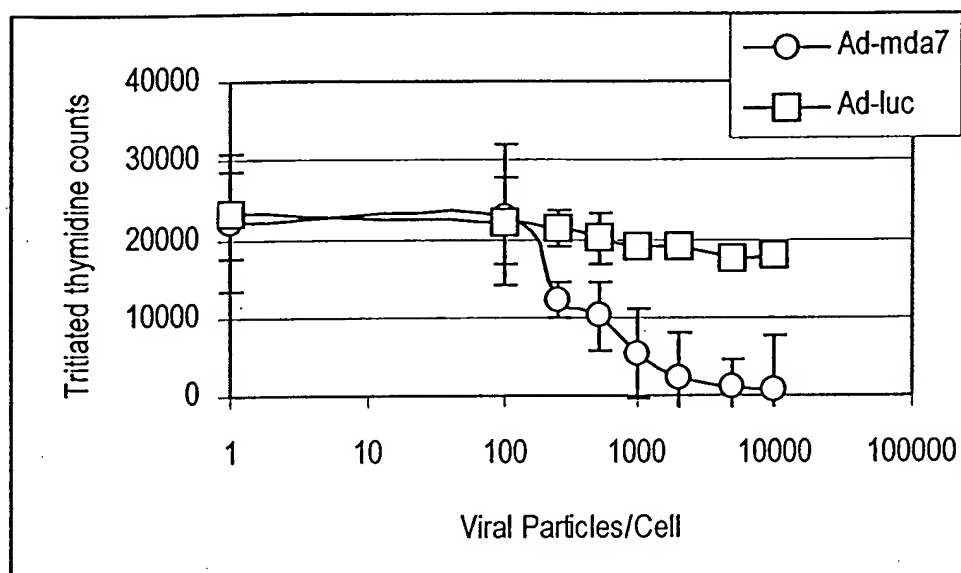
B   DLD-1 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)
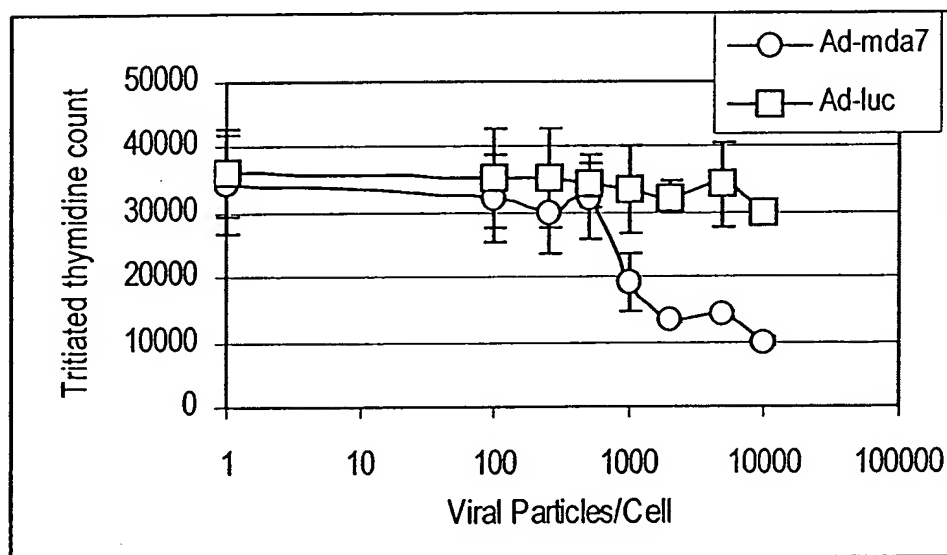
FIG. 6

A   MJ90 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)

B   HUVEC cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)

FIG. 7

FIG. 8

Control — 3.9%

AdLuc
(5000
vp/cell) — 4.0%

Ad-*mda*7
(1000
vp/cell) — 26.4%

Ad-*mda*7
(5000
vp/cell) — 57.4%

Annexin V staining
DLD-1 cells
Day 2

FIG. 9

(A) H1299 NSCLC Cells

(B) DLD-1 Colorectal Cells
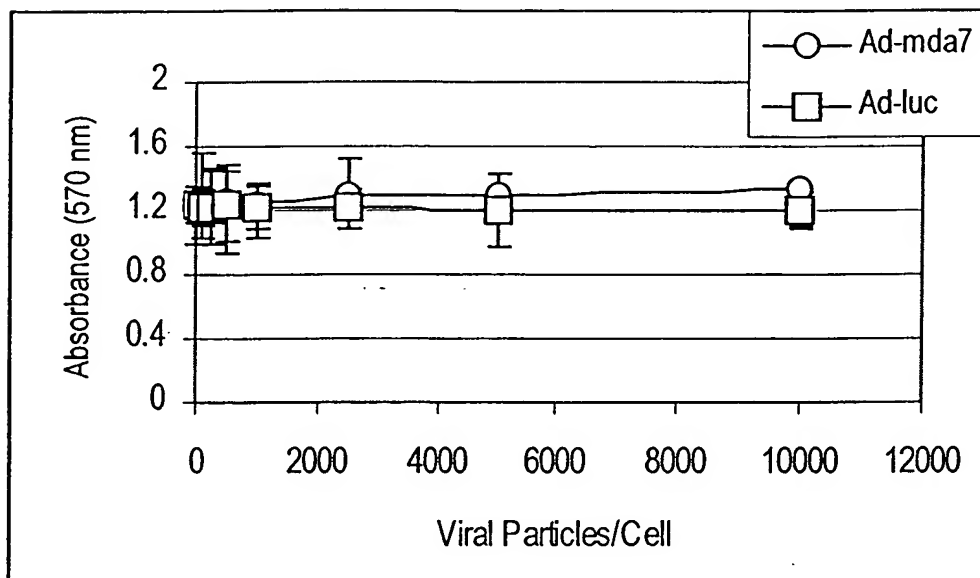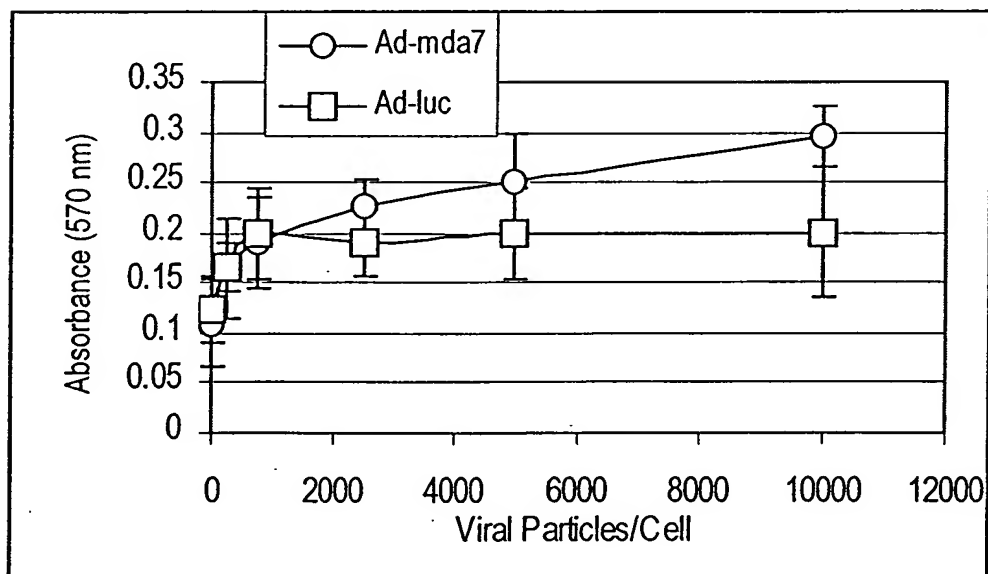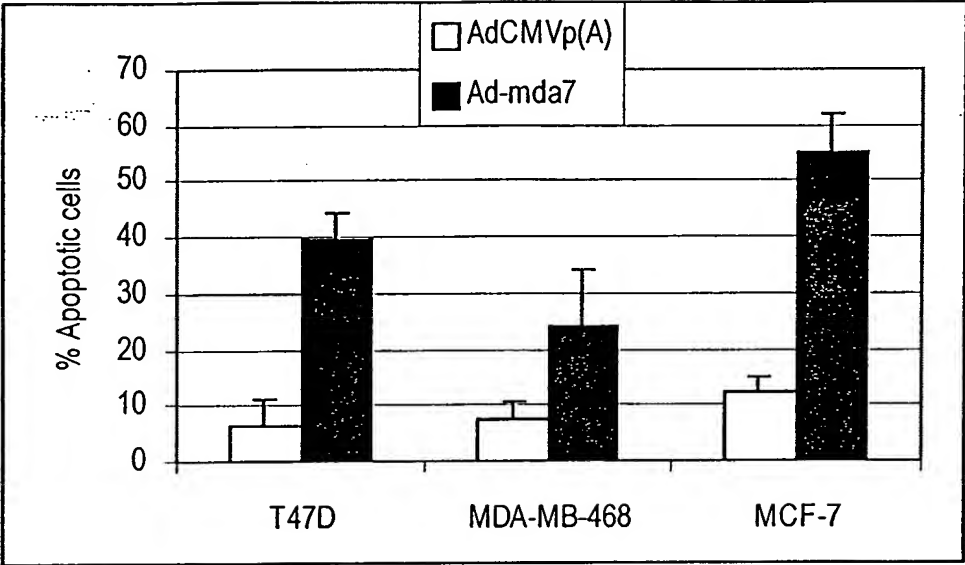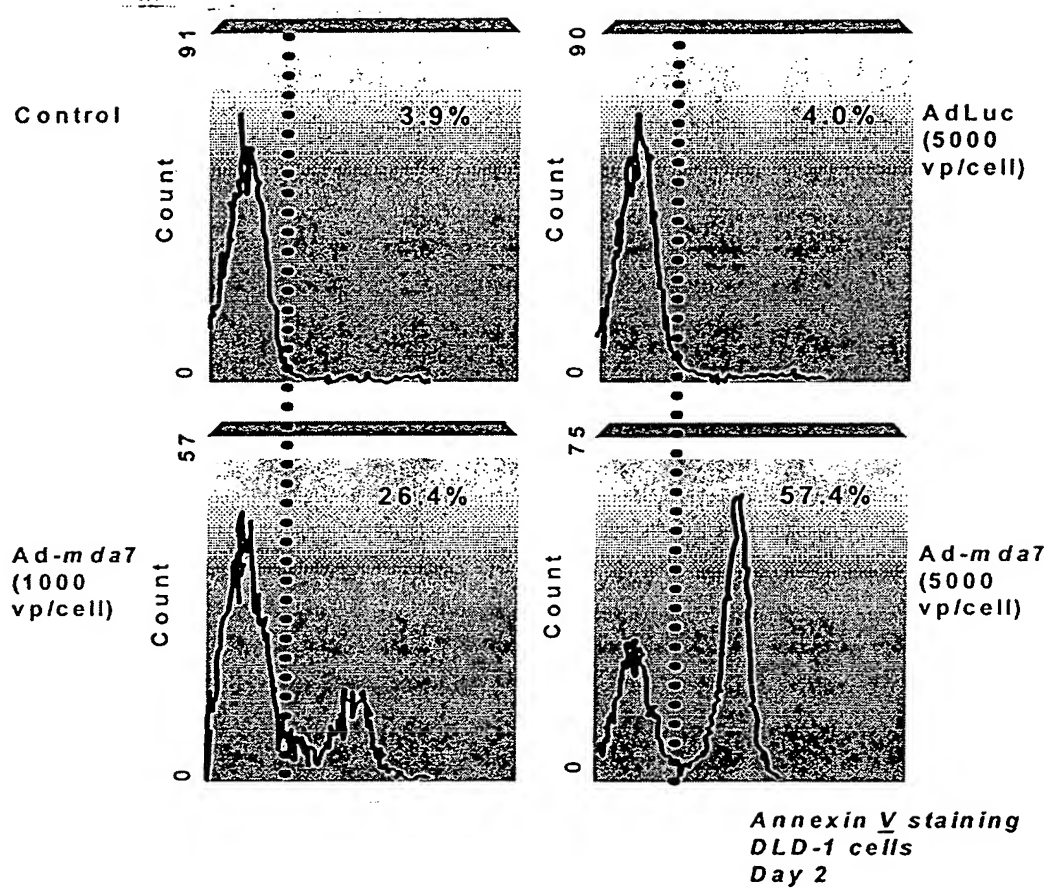
FIG. 10
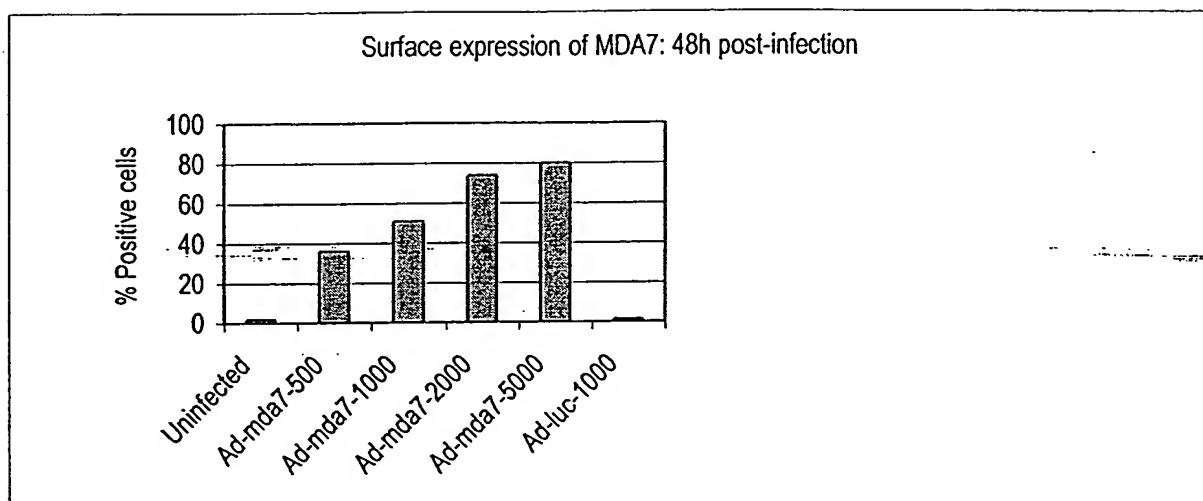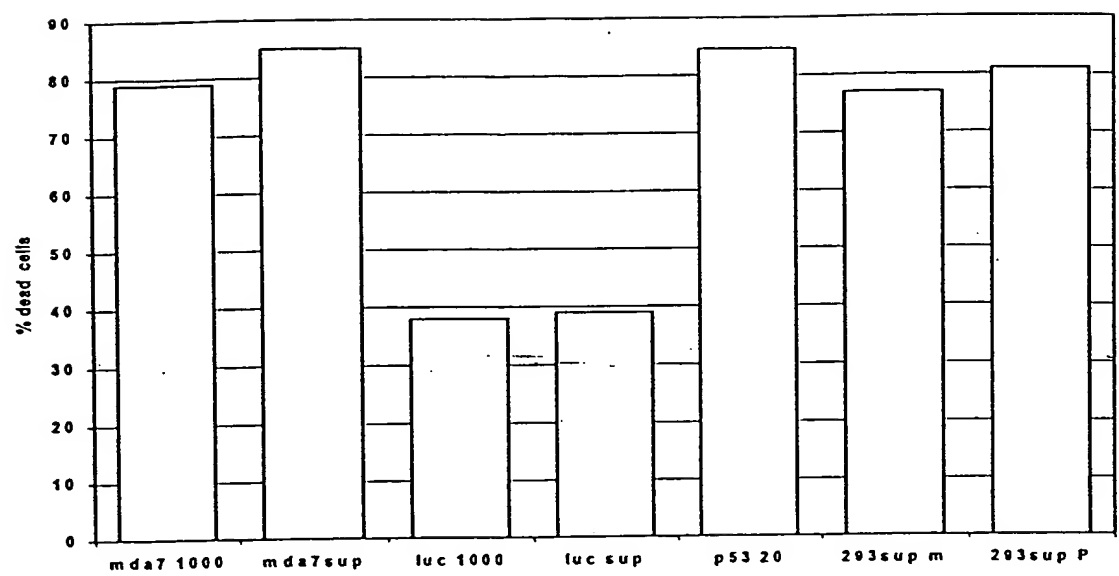
FIG. 11 A

FIG. 11 B
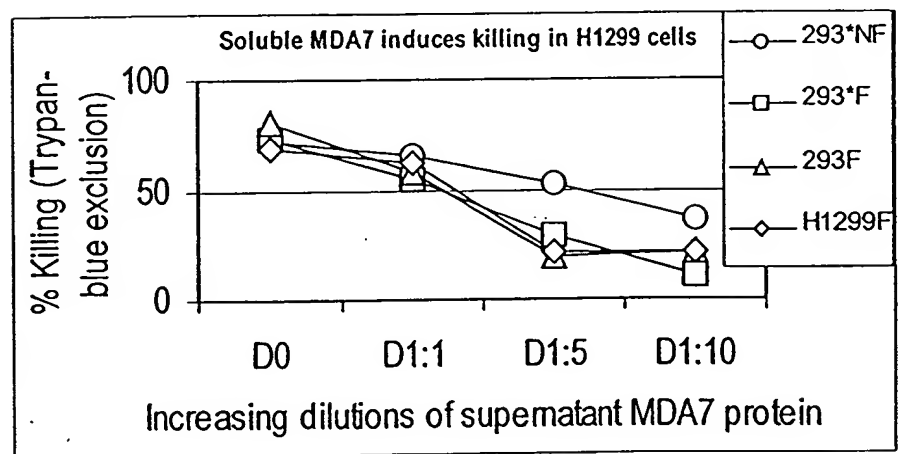
FIG. 12A

T47D against Ad-mda7+Tamoxifen (Simo):Day 4

Ad-mda7   Ad-Luc

Counts

60000
40000
20000
0

0/0   1.0/0   0/1000   1.0/1000

Tamoxifen(ug/mL)/ Vector(vp/cell)

MCF-7 against Ad-mda7+Tamoxifen (Simo):Day 4

Ad-mda7   Ad-Luc

Counts

60000
40000
20000
0

0/0   1.0/0   0/1000   1.0/1000

Tamoxifen(ug/mL)/ Vector(vp/cell))
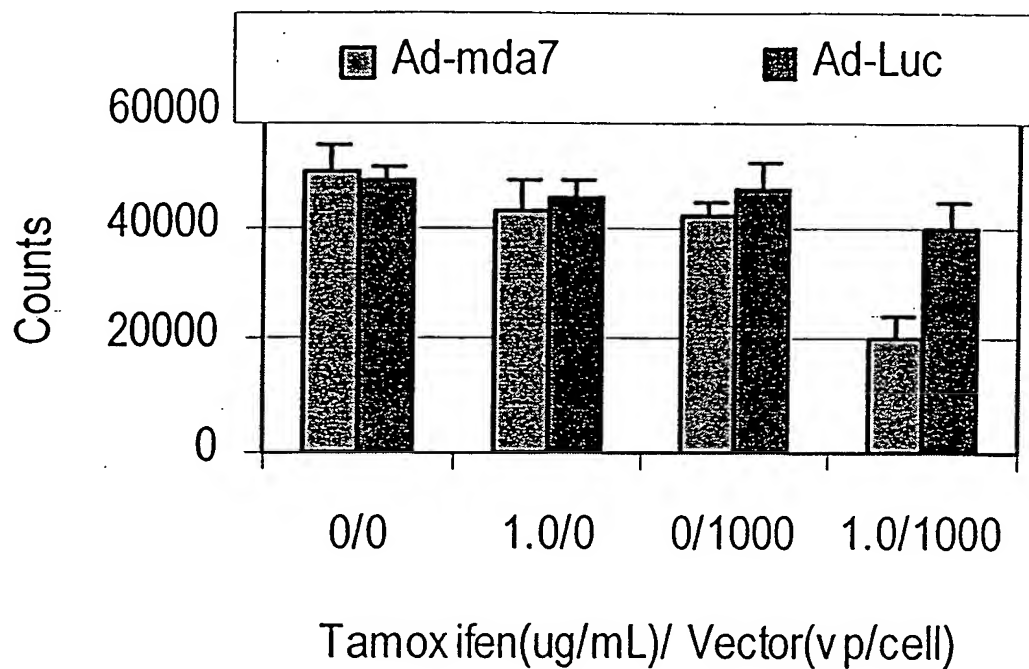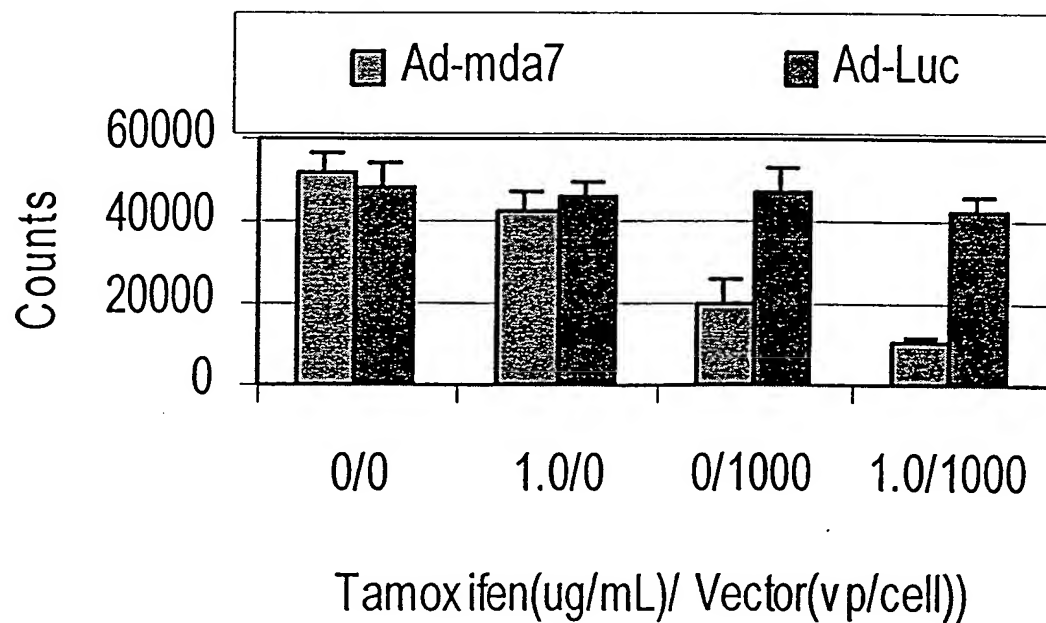
FIG. 12B

T47D against Ad-mda7+Adriamycin
(Simo): Day 3
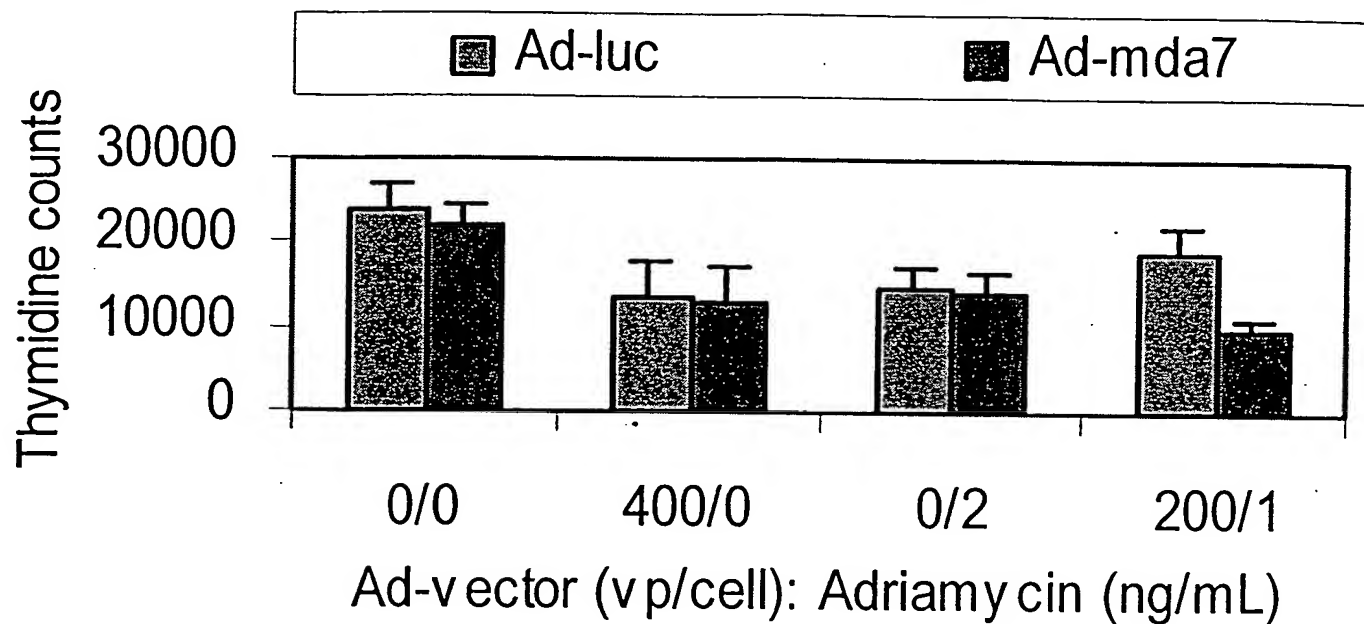
MCF-7 against Ad-mda7+Adriamycin (Simo):Day 3

FIG. 14

Cell Viability after Ad-mda-7 infection

Number of Viable Cells x10⁴

A549

H460

H1299

NHLF

Days after infection

PBS
Ad-Luc
Ad-mda-7
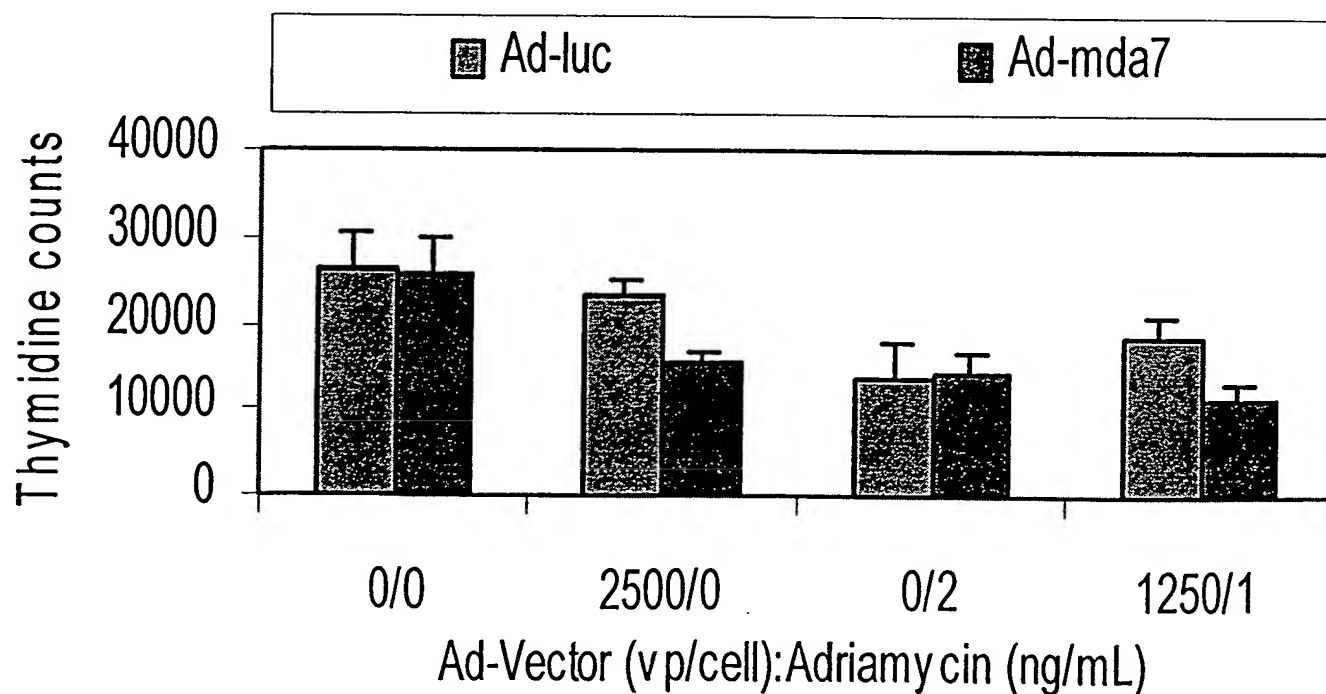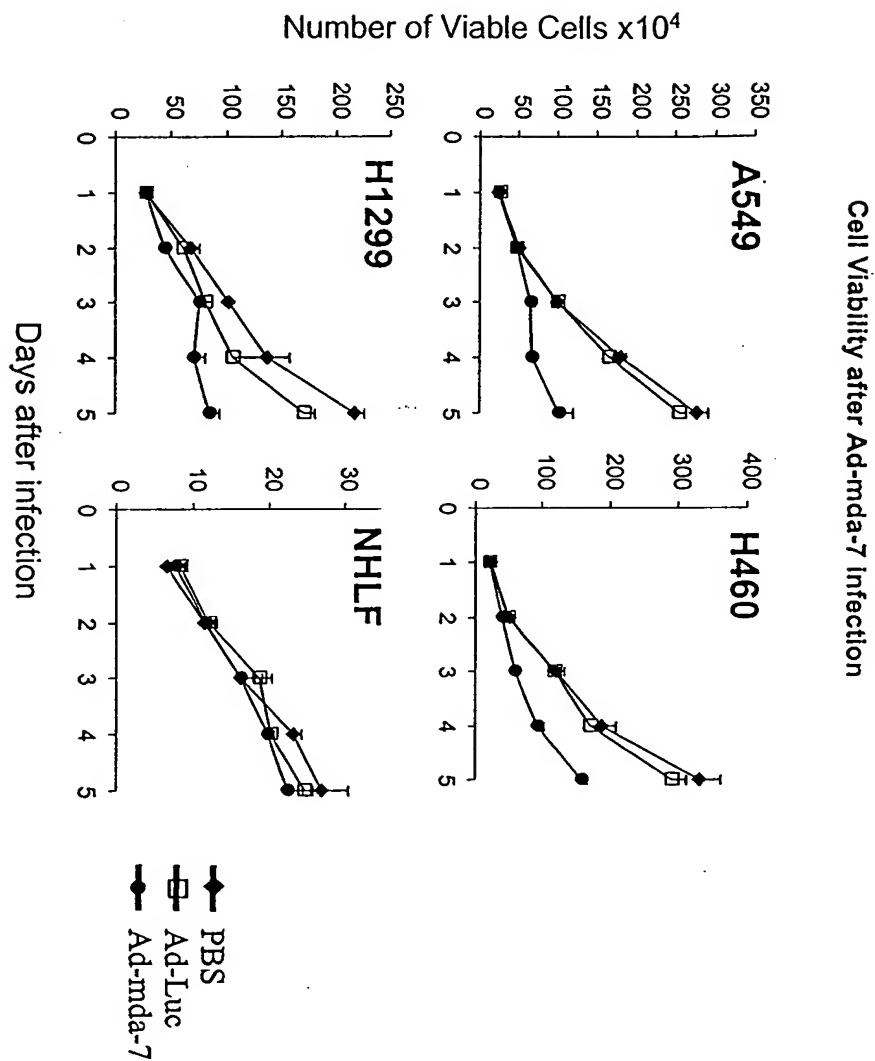
FIG. 15
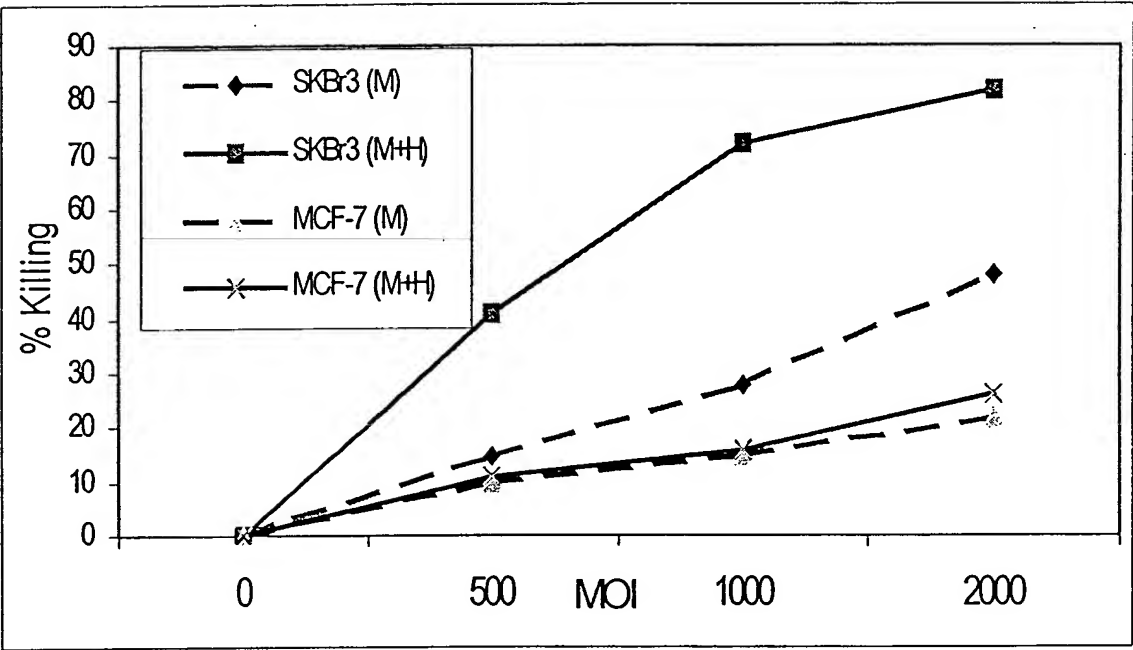
SKBr3   Her2 +
MCF-7   Her2 -
M   Ad-mda7
H   Herceptin

EXHIBIT 14